Monoclonal antibody studies on the prothoracic glands of Manduca sexta

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Frontispiece: SEM of PG cells from a fifth instar Manduca sexta larva, two days post wandering.

P = prothoracic glands; T = trachae.

The scale bar represents 100 microns

MONOCLONAL ANTIBODY STUDIES ON THE

PROTHORACIC GLANDS OF *MANDUCA SEXTA*

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Abbreviations

Ab	antibody
ACTH	adrenocorticortropic hormone
Ag	antigen
ANC	abdominal nerve cord
BB	borate buffer
BSA	bovine serum albumin
CA	corpora allata
cAMP	cyclic adenosine monophosphate
Ci	Curie
CNS	central nervous system
cpm	counts per minute
D	daltons
DAB	diaminobenzidine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FB	fat body
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthine aminopterin thymidine
20-HE	20-hydroxy ecdysone
HGPRTase	hypoxanthine-guanine phosphoribosyltransferase
HPLC	high performance liquid chromatography

HT	hypoxanthine thymidine
Ig	immunoglobulin
IP	intra peritoneal
JH	juvenile hormone
KD	kilo daltons
mCi	milli curie
2-ME	2-mercaptoethanol
mg	milligram
mins	minutes
ml	millilitre
mmol	millimole
mOsmoles	milliosmoles
mRNA	messenger ribonucleic acid
mvs	multivesicular sac
mwt	molecular weight
nm	nanometer
PAS	periodic acid Schiffs reagent
PBS	phosphate buffered saline
PCS	peripheral channel system
PEG	polyethylene glycol
PG	prothoracic gland
PSI	pounds per square inch
PTTH	prothoracicotropic hormone
PTU	phenylthiourea
PVC	polyvinyl chloride

RIA	radioimmunoassay
RPM	revolutions per minute
SAS	saturated ammonium sulphate
SDS	sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	scanning electron micrograph
secs	seconds
TEM	transmission electron micrograph
TLC	thin layer chromatography
ul	microlitre
um	micron
uv	ultra violet
W + 2	2 days post wandering

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Summary

The prothoracic glands of insects secrete a steroid hormone, ecdysone, which is hydroxylated in the peripheral tissues to form the active moulting hormone, 20-hydroxyecdsyone, that regulates moulting of the insect cuticle.

Several approaches were taken to study the structure and function of the prothoracic glands of Manduca sexta (L). All involved the application of hybridoma techniques.

Monoclonal antibodies were raised against a pure preparation of ecdysteroid coupled to a carrier protein. Both in vivo and in vitro immunisation protocols were used. The resulting monoclonal antibodies were characterised.

To investigate as yet uncharacterised molecules of structural and functional importance monoclonal antibodies were also generated using a crude prothoracic gland tissue preparation as immunogen. This resulted in the isolation of monoclonal antibodies which detected molecules in the connective tissue sheath which were partially characterised and found to be immunologically similar to vertebrate collagens. Monoclonal antibodies resulting from immunisation with PG tissue were also screened for anti-ecdysteroid activity. The monoclonal antibodies isolated by this strategy were characterised and possible roles in ecdysteroid secretion of the prothoracic gland antigens, which elicited the antibodies, were investigated.

The thesis also includes a discussion of Manduca endocrinology and theoretical and practical aspects of hybridoma technology.

Chapter I: The Hormonal Control of the Moult Cycle of the Tobacco Hornworm Manduca sexta

The insect species used throughout this study was the tobacco hornworm, Manduca sexta, (Lepidoptera Sphingidae). This chapter discusses some aspects of insect endocrinology and concentrates to the greatest extent on the hormonal control of the moult cycle in this species.

Manduca has many features of its development and morphology that make it an ideal experimental animal for the study of developmental processes. In practical terms it is large and has a rapid rate of growth. Emergence of the first instar larva from the egg to its ultimate pupation takes just 18-20 days, under optimal conditions with respect to light cycle, temperature and humidity. Manduca can be maintained under continuous culture and will conveniently feed, throughout larval life, on an artificial diet reducing the variation associated with and obviating the requirement for a supply of fresh tobacco leaves (Bell and Joachim, 1976). Once the animal has pupated the time taken before adult eclosion occurs is approximately 19 days under long day (17:7) conditions. Under a short day light cycle (12:12) the pupae enter a state of diapause and will not emerge for several months.

Developmentally Manduca is also an ideal experimental animal. In common with other holometabola there are three morphologically distinct phases in the life cycle (larval/pupal/adult). The control processes responsible for the initiation and maintenance of these stages can be investigated. For any developmental work it is of vital importance that the population of animals under study can be

synchronised very precisely at various phases of the life cycle. In Manduca this synchrony results from an endogenous circadian clock that precisely times the release of the prothoracicotropic hormone (PTTH) which, in turn, stimulates the prothoracic glands (PG) to secrete ecdysone (Truman, 1972). This effectively brings the developing animals into temporal alignment at various stages during their life history. The threshold for competence to secrete PTTH is bodyweight dependent (Nijhout and Williams, 1974). The release of PTTH is a gated process that only occurs for a few hours depending on the developmental stage and is a predictable event with respect to when the gate opens during the light cycle. The release of PTTH is therefore timed by a circadian clock which is entrained to photoperiod (Truman, 1972). Not all larvae will have reached the required weight threshold at the time the gate for PTTH release is open on a given day and will therefore have to wait until the gate is open on the following day to release PTTH. This is irrespective of whether or not they attain the weight threshold prior to the opening of the gate, PTTH can only be released during the hours the gate is open. This results in two populations of animals, those that had reached the weight threshold and were competent to release PTTH immediately the gate opened and the others that have to wait for the opening of the gate on the following day. These populations are designated as gate 1 and gate 2 animals respectively (Truman, 1972). Although there is variation within these two populations, because of the differential attainment of competence to release PTTH, it does mean the animals are synchronised to a great extent. The animals are also synchronised by the gating of PTTH release at the time of wandering and this allows the precise timing of fifth instar larvae. For reviews see Truman (1972) and Truman and Riddiford (1974).

When taken in combination these features of Manduca sexta make it the animal of choice for the study of many aspects of insect development.

Insect Hormones

Many of the processes essential for insect development are under hormonal control (for review see Richards, 1981). The two main classes of hormone responsible for the regulation of growth and development are the ecdysteroids and juvenile hormones (JH). These hormones also play a role in reproduction in adult insects along with some neurosecretory hormones. The maintenance of homeostasis along with the initiation of some behavioural events are under the influence of a number of neurosecretory hormones (Mordue, 1982).

The Insect Moult Cycle

The rigid exoskeleton (or cuticle) characteristic of insects and other arthropods prevents postembryonic development from following an uninterrupted course. For growth and metamorphosis to occur a new cuticle must periodically be formed and the old one discarded. Moulting begins with the separation of the epidermis from the old cuticle, a process termed apolysis. The intervening space between the cuticle and epidermis is filled with a moulting fluid, or gel, which contains a cocktail of protease and chitinase proenzymes. Following apolysis the epidermal cells undergo morphogenetic shape changes (cell division may occur at this time) and secrete plaques of new epicuticle at the tips of their microvilli. These plaques extend laterally and fuse to form the new epicuticle, which defines the shape and size of the next instar's cuticle. The insect is now encased in two layers of cuticle, the outer layer that will be shed at the next moult and an inner cuticle that is being actively formed

and will become the functional cuticle when the outer one is shed. The new cuticle grows in thickness as a new procuticle is formed, mainly by deposition of additional lamellae of chitin and protein at its inner surface, but also by intussusception of protein(s) within its thickness (see eg, Kaznowski et al, 1986). The enzymes of the moulting fluid are only activated relatively late in the moulting cycle, when the new procuticle is substantially complete. The enzymes in the fluids actively digest the old endocuticle, leaving the epicuticle to be cast as the exuvia, and the resulting products are resorbed along with the moulting fluid (for review see Jungreis, 1979). Within the exuvia are defined lines of weakness termed ecdysial sutures and it is these pre-weakened areas that rupture to allow the insect to escape from the exuvia. This rupture and subsequent escape is characterised by a complex behavioural repertoire that is only exhibited at the time of the moult (for review see Reynolds, 1980). Following ecdysis the new cuticle is expanded and sclerotised, this varies qualitatively with stage and species of insect. For a detailed discussion see Locke (1974; 1985); Anderson (1985) and Reynolds (1980; 1985).

Hormonal Control of the Moult Cycle

In the regulation of post embryonic insect development three hormones are generally considered to be involved, PTTH, ecdysone and JH. In 1922 Kopec demonstrated that the head was required for moulting and that a head-derived factor acted humorally to initiate the moult in the gypsy moth, Lymantria dispar. The localisation of the neurosecretory cells in the brain that synthesised the hormone was established by Wigglesworth (1934; 1940) by implantation experiments using small portions of the brain which were introduced into decapitated Rhodnius larvae. Recently the precise cellular

location of the PTTH synthesising cells has been pinpointed in Manduca to a single neurosecretory cell in each hemisphere of the brain amongst the lateral neurosecretory cells (Agui et al, 1979). In Manduca the release site of PTTH has been shown to be the corpus allatum (CA) (Agui et al, 1980) although under in vitro culture the corpus cardiacum (CC) also has the capacity for PTTH release (Carrow et al, 1981). Anatomical studies have demonstrated that the PTTH cells in the brain project only to the CC (Buys and Gibbs, 1981). The picture of the release of PTTH therefore remains unclear. There is some evidence however that in some species the neurosecretory cells may have the capacity to release PTTH directly into the haemolymph via the pars intercerebralis obviating the requirement for brain-CC/CA connections (Takeda, 1976). In the same insect, Monema flavesvens, some evidence is available that suggests that haemocytes may serve as carriers for PTTH and act as vectors of the hormone in the haemolymph (Takeda, 1977). This is supported by the fact that implanted brains devoid of CC/CA connections have the capacity to release PTTH. The precise site and manner of the release of PTTH in Manduca, under physiological conditions, remains to be established.

Chemical characterisation of PTTH from Bombyx mori suggested it to be a peptide (Ishizaki et al, 1977; Nagasawa et al, 1979; 1980). Partial amino acid sequencing of the PTTH derived from Bombyx mori heads has been reported (Nagasawa et al, 1984). Apparently Bombyx PTTH exists in two distinct mwt forms of 22KD and 4KD. At least three N-terminally distinct forms of the lower mwt PTTH have been isolated (Nagasawa et al, 1984). Astonishingly the determined N-terminal fragments are highly homologous with human insulin B-chain.

Whether PTTH structures are ubiquitous in the insecta or show

species differences has yet to be ascertained. An indication that species differences do occur, even within the Lepidoptera, has been shown by Bollenbacher et al (1984). In a study on Manduca PTTH two mwt forms of the neurohormone have been identified, 29KD and 6-7KD respectively, using bioassay systems to detect the presence of the hormone in gel filtration fractions. Perhaps the most significant finding in this study is the apparent differential action of these two mwt forms during development. The small mwt form is released early in the instar and induces the small ecdysteroid peak that elicits pupal commitment. The large mwt PTTH is released in a temporal manner that coincides with the large ecdysteroid peaks responsible for moulting. These are inferences drawn from the differing in situ bioassay activities of the two mwt forms. However it remains for direct evidence of differential roles for the two PTTH to be demonstrated.

It was shown by Wigglesworth (1934) and Fakuda (1940) that the PG were essential for insect moulting. Williams (1947; 1952) showed that the PG were the target tissue of PTTH. In response to the brain trophic hormone the PG secrete ecdysone which is a hormone essential for virtually all aspects of insect growth and metamorphosis (Wigglesworth, 1964).

Ecdysteroids

Ecdysone was isolated in 1954 from Bombyx mori (Butenandt and Karlson, 1954) along with small quantities of a more polar substance that proved to be β-ecdysone. It was not until 1965 that ecdysone was chemically characterised and found to be a steroid (Karlson et al, 1965). The molecule has eleven asymetric centres and was therefore prohibitively difficult to chemically synthesise. Even when successful synthesis was achieved the ecdysone was only produced

in very small quantities (Kerb et al, 1966; Harrison et al, 1966). However another source of ecdysone and related steroids was discovered to be plants and these steroids were termed phytoecdysones. In some ferns and other plants very large quantities of these steroids were isolated (for review see Williams and Robbins, 1968). All the phytoecdysones isolated to date have all shared a common basic structure and only exhibit differing chemistries in the structure of the side chain. The term now used to describe this family of related steroids is ecdysteroids. However ecdysteroids are not only found in insects and plants. It is now known that they can be detected in tissues from crustacea and other arthropods but also in molluscs (Romer, 1979) and some worms (Sauber et al, 1983; Mendis et al, 1983; Nirde et al, 1983). There is also evidence of ecdysteroids in coelenterates (Sturaro et al, 1982).

However despite the fact that ecdysteroids can be isolated from a variety of invertebrate groups the steroids have not been shown in all cases to be endogenously synthesised and could be dietary in origin.

Ecdysone is the immediate precursor of β-ecdysone which is the 20-hydroxy derivative (Goodwin et al, 1978) and is therefore more commonly termed 20-hydroxyecdysone (20-HE). Using bioassay systems it has been demonstrated that 20-HE is approximately one hundred times more biologically active than ecdysone (Ashburner, 1971) but it is ecdysone that is secreted by the PG (Chino et al, 1974; King et al, 1974). However the PG lack the competence to hydroxylate the steroid to its 20-hydroxy form. Hydroxylation occurs in a range of peripheral tissues which possess the enzyme required to hydroxylate ecdysone at the C-20 position (King and Siddall, 1969; Hoffmann et al, 1975; Bollenbacher et al, 1975). In Manduca it has been reported that only the PG are able to secrete ecdysone (King et al,

1974) although some studies in other species have implicated oenocytes as a potential source of the steroid. Indirect evidence was reported by Locke (1969). The study demonstrated that oenocytes have ultrastructural similarity to steroid secreting cells. Romer et al, (1974) demonstrated in vitro ecdysone secretion by isolated oenocytes from Tenebrio molitor. However the function of oenocytes in Manduca remains to be established. It is known that oenocytes function as a source of cuticular paraffins in the desert locust (Diehl, 1973). Chadwick (1955) reported that in the absence of PG roaches would moult in an apparently normal manner. Similar findings have been reported in Galleria (Slama, 1983). The true physiological significance of these findings remains to be ascertained. In intact normal animals there is a body of evidence to suggest that the PG are the most significant physiological source of ecdysone. Changes in the ultrastructure of the PG also show a temporal correlation with ecdysone titre in Manduca sexta (Sedlak et al, 1983). There is also a demonstrable difference in the ultrastructure of active and inhibited PG in other species (McDaniel et al, 1976).

Biosynthesis of Ecdysone by the PG

For a full discussion of ecdysone biosynthesis see Rees (1985). It was first shown that steroid hormones are derived from cholesterol in 1945 (Bloch, 1945). Radiolabelled cholesterol was fed to a pregnant woman and radiolabelled pregenadiol was excreted in the urine. By comparison with vertebrate steroids the ecdysteroids differ significantly by virtue of having retained the full C27 carbon skeleton of cholesterol from which steroids are derived. They also have several hydroxyl groups at different sites on the molecule rendering them relatively water soluble. The

structural similarities between cholesterol, ecdysone and 20-HE are illustrated in Fig 1.1.

The elucidation of the biosynthetic pathway of ecdysone is far from being complete and one problem has been the low endogenous levels of many of the putative intermediate molecules involved. The situation is further exacerbated by the high endogenous levels of the precursor cholesterol which hinders the detection of intermediates present in small quantities (see Rees, 1985).

Insects, unlike the vertebrates and plants, lack the capacity to synthesise sterols from acetate or mevalonate. Consequently a dietary source of pre-formed sterol must be available or be provided by intestinal micro-organisms. It should be stressed that only a small component of the dietary sterol is actually utilised for ecdysone synthesis, the greatest part being incorporated into membranes, etc. The nature of the sterol depends on the insect's diet with phytophagous species ingesting it in the form of sitosterol or stigmasterol which are subsequently converted predominantly into cholesterol. Carnivorous species ingest pre-formed cholesterol. The details of the dealkylation of sitosterol to cholesterol in the phytophagous Manduca sexta are discussed by Svoboda and Robbins (1967; 1968). The use of tritiated cholesterol to monitor the conversion of cholesterol to ecdysone was first reported in the fly Calliphora erythrocephala; successful in vivo conversion was observed (Karlson and Hoffmeister, 1963). The PG are generally considered to be the site of ecdysone biosynthesis but the glands do not store the hormone to any appreciable extent as measured by RIA, etc (Agui et al, 1972; King et al, 1974; Chino et al, 1974; Bollenbacher et al, 1976; Hirn et al, 1979). However, the evidence of the PG being the site of ecdysone biosynthesis prior to successful in vito culture of PG was indirect, being the result of

extirpation and implantation experiments. For the results of *in vitro* studies with *Manduca* PG see King *et al* (1974). From this data, along with *in vitro* studies with the PG of other species (Chino *et al*, 1974; Hirn *et al*, 1979; Bollenbacher *et al*, 1976) it appeared that low levels of ecdysone in the PG could be attributed to ecdysone being secreted immediately following synthesis. King *et al* (1974) further stated the PG to be the sole source of ecdysone in *Manduca* as no other tissues could be found to secrete detectable levels of ecdysteroid. These findings are consistent with the PG being the major source of ecdysone in larval *Manduca* (the glands degenerate shortly after adult eclosion) and that on release into the haemolymph the ecdysone is transported to the peripheral tissues where the C-20 hydroxylation occurs and the more biologically active 20-HE results. In a sense this ecdysone to 20-HE conversion is a step in the metabolism of the steroid rather than its biosynthesis if a strict definition of a hormone is borne in mind, that is 20-HE is a biologically active metabolite. It is not yet apparent whether ecdysone or 20-HE is the form of the hormone that is inactivated. Because the conversion of ecdysone to 20-HE is the only step in the cholesterol to 20-HE pathway that is at least partially understood, in terms of enzyme systems, it merits brief consideration.

The study of ecdysone to 20-HE conversion was facilitated when radiolabelled ecdysone of high specific activity became available (King and Siddall, 1969; Hafferl *et al*, 1972). The reaction involves a single hydroxylation leading to the generation of the more biologically potent 20-HE. The enzyme responsible is ecdysone 20-mono-oxygenase (for review see Smith, 1985). In *Manduca* subcellular localisation was achieved in FB cells (Bollenbacher *et al*, 1977). Approximately 70% of the enzyme activity was localised in the mitochondrial fraction with the remainder being found in the

microsomes and nucleus. It is considered that the enzymic conversion of ecdysone to 20-HE is similar to vertebrate steroid hydroxylation (Gower, 1974). For further details on the conversion of ecdysone to 20-HE see the review by Smith (1985).

Control of the Temporal Release of Ecdysone by the PG of Manduca sexta

Of the various ecdysteroids that can be isolated from the tissues of Manduca it is commonly believed that ecdysone and 20-HE are of most physiological importance. The other ecdysteroids are considered to be precursor molecules or degradation products although the precise role of ecdysteroid conjugates has yet to be established (for review see Smith, 1985).

From the work of Ashburner (1971) it was demonstrated that in an in vitro assay using puffing of polytene chromosomes of Drosophila melanogaster, 20-HE was approximately one hundredfold more biologically active than ecdysone. It is ecdysone and 20-HE that regulate the insect moulting cycle. Recently the temporal changes in ecdysteroid titer in Manduca sexta have been measured (Bollenbacher et al, 1981; Riddiford, 1981) see Fig 1.2. Although only the profile of ecdysteroid titers for Manduca will be discussed the temporal pattern is similar for many other insect species (for review see Smith, 1985).

At each moult, be it larval-larval, larval-pupal or pupal-adult, there is a peak in ecdysteroid levels just prior to the moult. There is also a small peak of ecdysteroid just prior to Manduca entering into the wandering phase (Bollenbacher et al, 1975). In the final larval instar of Manduca it is thought that the PG are the sole source of detectable ecdysteroid (King et al, 1974). This is supported by the evidence that the whole body titer of ecdysteroid

can be accounted for by the amount of ecdysone produced by the PG under *in vitro* culture conditions (Stott, 1983). Some of the factors that regulate the ecdysteroid titer so precisely will now be discussed.

Prothoracic Gland Stimulation

The activation of the PG results from the release of PTTH from the CC/CA (Carrow *et al*, 1981) (The release site of PTTH in *Manduca* has been discussed elsewhere in this chapter). Although PTTH can be detected in the haemolymph at the time of its release (Gilbert *et al*, 1981) this does not in itself preclude the possibility of other, as yet unidentified, vectors of the neuropeptide. Because the severing of nervous connections to the PG of *Galleria mellonella* (L) does not appear to adversely affect ecdysone secretion this does not seem to be important in gland stimulation (Granger, 1978). PTTH stimulation of the PG is the result of direct action on the glands and once activated the basal rate of ecdysone secretion increases (Bollenbacher *et al*, 1979). It has also been reported that both PTTH and the JH analogue ZR515 could activate the PG in *Mamestra brassicae* (Hiruma *et al*, 1978). The capacity of JH to stimulate the glands was restricted in a temporal manner to the final part of the last larval instar and to the pupal phase. Another interesting observation in the same study was the apparent capacity of the PG to lose their competence to respond to JH and PTTH if maintained in the absence of the two hormones for long periods of time. Although this final observation is of only limited physiological interest it does pose interesting questions about the receptor systems used by the PG to detect these two hormones. The study of PG regulation used an *in vivo* system and involvement of JH was considered to be by direct action on the PG (Hiruma *et al*, 1978; Cymbrowski and Stolarz, 1979; Safranek *et al*, 1980; Hiruma and Agui, 1982). An *in situ* and *in vivo*

approach was taken by Greutzmacher et al (1984b) who considered the possibility that JH activation of the PG could be an indirect system. Evidence was provided that JH does not exert its stimulatory effect on the PG directly (in contrast to PTTH) (Greutzmacher et al, 1984a). In this study a stimulatory factor derived from FB of 30KD was implicated as the substance that acts on the PG under the influence of JH. Pursuing this investigation of secondary regulatory factors of PG activity Watson et al (1985) demonstrated the existence of a haemolymph stimulatory factor, also of 30KD, which promoted heightened ecdysone secretion in vitro in larval and pupal PG of Manduca sexta. It has been suggested that the haemolymph factor is the blood borne form of the FB factor (Watson et al, 1985). Watson et al (1985) suggested a hypothesis that could explain the interaction of JH, stimulatory factor(s) and PTTH in the activation of the PG during development (see Fig 1.3).

It is envisaged that during the fifth larval instar of Manduca the fall in JH titer just prior to the animal wandering, and becoming committed to pupal development, causes a drop in secretion rate of the factor from the cells of the FB. On day 3 or 4 of the instar PTTH is released and it is postulated that the low levels of the FB factor limits the responsiveness of the PG to PTTH leading to only a subtle rise in ecdysteroid titer (commitment peak). It should also be mentioned that the molecular form of PTTH released is also a factor that influences the state of activation of the PG (Bollenbacher and Gilbert, 1981; Bollenbacher et al, 1984). It is therefore speculated that only PTTH influences the peak in the ecdysteroid titer that occurs on day 3-4 of the fifth instar. After a temporary absence of detectable JH at this time the titer rises on day 5-6 (Granger et al, 1982). The commitment peak of ecdysone acts on the brain which then promotes JH release from the CA (Whisenton

et al, 1985). The rise in JH titer stimulates an increased release of stimulatory factor from the FB into the haemolymph which promotes ecdysone secretion by the PG on days 5-7 of the instar. By the time the second pulse of PTTH is released (day 7-8) in combination with saturating levels of the stimulatory factor, the ecdysteroid level rises to its maximal titer by day 8. This ecdysteroid peak directly precedes pupation.

Whether or not this partially hypothetical scheme for the control of PG secretion of ecdysone during the fifth instar of Manduca is verified the evidence to date strongly suggests a hitherto unforeseen complexity of PG control. The combined influence of PTTH and the stimulatory factor and the evidence that they can work independently of one another provides a mechanism of control of PG secretion both in terms of hormone titer and the temporal pattern of release.

As to the actual nature of the 30KD stimulatory factor it has been speculated that it could serve as a sterol precursor transporting protein (Watson et al, 1985). It is thought that the precursor could be a more oxygenated molecule than cholesterol (Bollenbacher et al, 1979b), possibly the immediate precursors of ecdysone, 2,22-dideoxyecdysone or 2-deoxyecdysone (for review of ecdysone biosynthesis see Rees, 1985). Whereas PTTH acts on the PG via CAMP (Vedeckis et al, 1974; 1976) the evidence suggests that the stimulatory factor apparently acts independently of CAMP (Smith et al, 1984). Reliable information on the mode of action of the factor will only be obtained when its purification and chemical characterisations have been achieved.

Apart from the control of PG secretion the mechanism(s) responsible for the termination of the PG activity must also be important. The 'switching off' mechanisms are not understood but

apart from hormonal involvement it is possible that the extensive nervous innervation of the PG, which connect to the suboesophageal ganglia as well as the prothoracic ganglia and mesothoracic ganglia, are also important (Stott, 1983). The possible role of nervous innervation in the control of PG secretion remains controversial.

Metabolism and Excretion

Another two important factors in the reduction of circulating ecdysteroids are of course metabolic inactivation and excretion. The balance between these events on the one hand and biosynthesis and secretion on the other will control the overall ecdysteroid titer at any given time. The major tissues involved in the metabolism of ecdysone and 20-HE are the FB, malpighian tubules as well as the mid-gut. The study of ecdysone metabolism utilises radiolabelled ecdysone and the subsequent tracing of the label to the compounds resulting from metabolism. The two major classes of product isolated by this technique are both highly polar and consist of carboxylic acids and conjugates. The carboxylic acids are the products of C-26 hydroxylation followed by subsequent oxidation. The conjugates are believed to be sulphate (Koolman et al, 1973) or phosphate esters (Isaac et al, 1982) glucosides and glucuronides (Heinrich and Hoffmeister, 1970).

The conjugates differ functionally as well as structurally with some being inactivation products while others are considered to be storage forms of the steroid (for review see Koolman and Karlson, 1985). The extent to which metabolism affects the ecdysone titer during insect development is difficult to study. The time course of the appearance and disappearance of some ecdysone metabolites have been followed successfully in larval Calliphora vicina (Karlson and Koolman, 1973; Koolman, 1981) and larval Locusta migratoria (Hoffmann

et al, 1974). There is a general consensus that the levels of metabolites fluctuate during insect development and the assumption is that this results from physiological regulation. There is evidence from in vitro studies that the decrease in ecdysone titer in short term studies is mirrored by increasing levels of some metabolites (Koolman, 1981). For a review of the enzymes involved in ecdysteroid metabolism see Koolman and Karlson (1985). A simple diagram of the sites of reactions on the ecdysone molecule which are considered to be of most significance in metabolism is given in Fig 1.4. Most commonly the metabolic products from ecdysone are excreted by the insect along with a certain amount of free ecdysone. The main structures responsible for the excretion are considered to be the malpighian tubules and the gut. In Manduca larvae, active transport across the gut wall is probably the major route of excretion (M. M. A. Jones, personal communication). In developmental stages where this method of disposal of ecdysone and its metabolites is impossible, such as eggs, embryos and pupae, other methods are used to inactivate the enzymes or by confining the metabolites to storage sites such as pupal meconium produced in Lepidoptera (see Kaplanis et al, (1980) for a discussion of Manduca sexta). Another factor that could be important in the regulation of ecdysone titer is the presence in the haemolymph of specific ecdysteroid binding proteins. Blood proteins that bind steroids and protect them from enzymic degradation are well known in vertebrates. Although evidence for the existence of such binding proteins is available for some insect species (Feyereisen, 1977; 1980; Emmerich, 1970a; 1970b; Butterworth and Berendes, 1974; Thamer and Karlson, 1972; Reum et al, 1982) there is little consensus on their importance in regulating ecdysteroid titers. Little is known about the occurrence of such proteins in Manduca.

Ecdysteroid Receptors

Following secretion by the PG, ecdysone circulates in the haemolymph from which it has access to peripheral target tissues (see above). The cells sensitive to the steroid modify their cellular activity in response to the hormone in a manner dependent on the type of tissue responding and the stage of development. The cells detect ecdysone through the binding of the hormone to cellular receptors which are presumably proteins. Control of sensitivity to the hormone might be influenced by differential receptor number. This would require the target cells to synthesise and degrade receptors as appropriate. For a discussion on ecdysone receptors see Yund and Osterbur (1985). Tissue specificity exhibited during development and the differential responsiveness by the tissues must be due to cellular events as the hormone remains structurally constant throughout development. Perhaps different receptors are responsible for the differential specificity observed. The major studies on receptors have utilised Drosophila imaginal discs and Drosophila KC cells as a source of tissue (Bonner, 1982; Maroy et al, 1978; Sage et al, 1982).

Studying receptors has traditionally involved the use of radiolabelled hormone to bind the receptor. Alternatively the hormone with an attached photoaffinity group is allowed to interact with its receptor and then exposed to a wavelength of light which is photoactive. The resulting photoactivated group covalently binds to the receptor. Unlike the radiolabelled hormone binding technique in this case the hormone-receptor association is irreversible. Obviously this does not closely parallel hormone-receptor interactions under physiological conditions. However this has proved a useful technique for studying Drosophila ecdysone receptors (Shaltmann and Pongs, 1982). Although the two techniques have been

useful tools for the study of hormone-receptor interactions in target tissues, another very powerful approach to the study of receptors, so far not applied to insects, is the use of Abs raised against purified receptor preparations. Monoclonal antibodies (MAb) against crude tissue preparations are potentially very useful because affinity column purification of the receptor is then possible and obviates the requirement for a pure receptor preparation. One such example is the purification of the β-adrenergic receptor using MAb (Fraser and Venter, 1980). This approach has obvious advantages in the study of insect receptors where the quantities of available tissue is invariably small. Ab is also useful in investigating receptors that are not in an active state and can therefore be potentially more sensitive than the radiolabelled technique described.

There are two components of the target cell that have receptors for ecdysteroid, the cytosol and the nucleus (Emmerich, 1972; Hill et al, 1982). The relevance of these two locations is discussed below.

Ecdysteroid Mode of Action at the Molecular Level

It was the relationship between high ecdysone titers and the resulting specific transcription by polytene chromosomes in the form of chromosome puffs that led to the first hypothesis of how steroids exert their action at the molecular level in target cells (Clever and Karlson, 1960; Karlson, 1963). In 1968 a similar theory was postulated by Jensen et al (1968) for vertebrate steroid action. The basic mode of action in both invertebrate and vertebrate systems was thought to be the same and can be summarised as follows.

Steroids in the circulatory system would cross the cell membrane of target cells and encounter specific cytoplasmic receptors to which the steroid would bind. The resulting steroid-receptor complex would then enter the cell nucleus and would bind to the chromatin at specific sites and in this way influence gene expression. These hypotheses were strongly influenced by the work of Jacob and Monod (1964) on the induction of lactose-metabolising enzymes in E. coli in the presence of substrate, which had been discovered to be a process of derepression. Thus, ecdysteroid-induced genes were hypothesised to be similarly derepressed by the binding of the ecdysteroid-receptor complex to an operator site. As to whether or not the gene activation brought about cellular changes directly or indirectly was not known. The further elucidation of steroid mode of action progressed much faster for vertebrate steroid hormones (eg, the action of progesterone on the oestrogen-primed chick oviduct - O'Malley and Shrader (1976)) than for ecdysteroids, doubtless due to the greater resources deployed for the former work. However, the paradigm established for vertebrate steroids has been followed in work on ecdysteroids in recent years. Most of this work has used either imaginal discs mass-isolated from Drosophila or Drosophila cell lines (KC or tumorous). Data from KC cells suggests that the entry of ecdysteroids into target cells is by diffusion rather than some sort of facilitated transport mechanism (for review see O'Connor, 1985), although Spindler and co-workers (see Spindler et al, 1984) have shown that there is an active uptake process for ecdysteroids in crustacean epidermis, it has been demonstrated conclusively that saturable binding sites for ecdysteroids with receptor-like properties exist in the tissues of insects and

crustacea (Yund et al, 1978; Maroy et al, 1978; Gronemeyer et al, 1981; Kuppert and Spinder, 1982). The receptors occur both in the cytoplasm and nucleus of target cells (eg, Hill et al, 1982). It is still not clear, even for vertebrate steroid receptors, if cytoplasmic receptors are real or artefactual (see for example, King and Greene (1984) and Welshons et al (1984)). However evidence in favour of the proposition that 20-HE binds cytoplasmic receptors and this complex then migrates to the nucleus has been produced (eg, Schaltmann and Pongs, 1982). There still remains one very fundamental question in this scheme of events, does the ecdysone become hydroxylated to 20-HE in target cells? Evidence from Drosophila imaginal discs suggests not (Silvert and Fristrom, 1980) but there is apparently target cell hydroxylation in the imaginal discs of Pieris (Blais and Lafont, 1980). Once bound to its cytoplasmic receptor there is an indication from vertebrate studies that the complex shows an increased affinity for DNA during the cytoplasmic to nuclear transition (Litwack et al, 1980). Direct binding by the receptor-20-HE complex to the chromatin has been demonstrated in chromosome puff studies (Gronemeyer and Pongs, 1980) and in Manduca epidermal cells (Dyer and Riddiford, 1983). Once bound, the ecdysteroid receptor complex presumably modulates transcription in order to produce characteristic effects of the hormone. Ecdysteroid action has been shown to involve both increased and decreased levels of expression of particular gene products (eg, Riddiford, 1984). Ecdysteroid is required at several stages if correct gene induction is to take place as a result of chromatin binding (Ashburner and Richards, 1976; Richards, 1985). The ecdysone induced protein synthesis is initiated before any morphological changes in the cells can be observed both in salivary glands and KC cells (Savakis et al, 1980; O'Connor, 1985).

Ashburner et al (1974) postulated that in salivary glands early puffs may produce at least one protein which functions to activate subsequent synthesis of later RNAs but interestingly also inhibits its own synthesis, that is, acts to control gene expression. Perhaps this is a general phenomenon in ecdysone inducible gene systems.

Ecdysteroid Mode of Action at the Cellular Level

Ecdysteroids exert their influence on moulting by acting on epidermal cells and thereby control the production of cuticle. This system is relatively well understood in Manduca (Riddiford, 1982; Riddiford, 1985). The effect of JH on this system is of paramount importance during the final embryonic phase and throughout larval life and will be discussed in detail later in this chapter. An outline of the morphological events that characterise moulting has already been given, but how do these events relate to ecdysteroid titers?

At the time of a moult the ecdysteroid titer rises and the cells of the epidermis respond by heightened RNA and protein synthesis and the cellular shape may alter. It is reported that the exposure time to ecdysteroid necessary to elicit these events is often relatively short and different areas of the epidermis show differential ecdysteroid requirements (Mitsui and Riddiford, 1978). These events are reviewed in detail by Fristrom et al (1982). DNA synthesis is required for subsequent mitosis and the extent of DNA synthesis varies according to species and also is dependent upon the type of moult. For instance during the final larval moult the extent of DNA synthesis is much less than during the larval-pupal or pupal-adult moult in Galleria mellonella (Sehnal and Novak, 1969). This can probably be attributed to the requirement for new cuticular structures when the insect undergoes metamorphosis. During the pupal

moult in Manduca there is evidence that one round of DNA synthesis is temporally correlated with the first release of ecdysone (Wielgus et al, 1979). However this DNA synthesis is not obligatory for developmental reprogramming and in the absence of DNA synthesis at this time the subsequent pupal gene expression is not impaired (Dyer et al, 1981).

At a larval-larval moult there is a detectable reduction in mRNA and protein responsible for endocuticle production in response to the rise in ecdysteroid titer. It is considered that the cells are then directing their resources to the production of moulting fluid and do not resume endocuticular protein synthesis until ecdysis or just after ecdysis (Riddiford, 1982).

Prior to the larval-pupal moult in Manduca there is the small commitment peak of ecdysteroid in the absence of detectable JH (see Fig 1.2). Again transcription of mRNA coding for larval endocuticular proteins ceases and after an intervening period of some 72 hours mRNA for pupal cuticular proteins becomes detectable in response to the second ecdysteroid peak, some 24 hours before ecdysis. This change only occurs if JH is absent. How JH acts at the molecular level to block the reprogramming action of ecdysteroid is unclear. The control of 20-HE induced gene activation in this phase of the moult cycle is not well understood. However it could well be analogous to the system postulated by Ashburner et al (1974) for Drosophila salivary gland chromosome puffs. This would then suggest that ecdysone induces a regulatory protein(s) early in the response that induces subsequent RNA synthesis and also regulates its own expression. The hormone that induces these cellular changes is 20-HE (eg, Marks, 1980) although in some species it may be ecdysone (Blais and Lafont, 1980). In 20-HE sensitive species the anatomical site of hydroxylation is unclear and may be in the target

cells themselves in some species but elsewhere in others (see previously, and for a review Riddiford, 1985). There is evidence for differential response to ecdysteroid by epidermal cells located in different body regions. In chick oviduct cells initial exposure to oestrogen causes some cells to synthesise increased number of receptors (Mulvihill and Palmiter, 1977). A similar system could be envisaged in insect epidermal cells leading to a population of late-responding cells which had synthesised receptors in response to initial 20-HE exposure rendering them competent on second exposure to the steroid. Indirect evidence is available for this (Dyer and Riddiford, 1983).

Although there are some species differences, generally, the rising ecdysone titer and the event of apolysis correlate well. By the time the peak titer is achieved apolysis is complete (Riddiford, 1985). Similar correlations also occur where new cuticle is deposited and occurs either at the peak of the ecdysteroid titer or as it begins to decline (Kiguchi and Agui, 1981). There is evidence from *in vitro* studies that the hormone responsible for the deposition of cuticle is 20-HE rather than ecdysone (for review see Oberlander, 1980). There is more good evidence that in many, if not all insects events late in the moult cycle (ie, continued cuticle deposition, pre-ecdysial tanning, moulting, fluid activation and resorption, and ecdysis) are dependent on a reduction in the ecdysteroid titer. For example, Fristrom *et al* (1982) demonstrated prolonged exposure of *Drosophila* imaginal discs to 20-HE inhibited procuticle deposition implying decreasing titer is important for normal cuticle formation. The falling titer of ecdysteroid is also important in the tanning of some cuticular elements prior to the completion of ecdysis (eg, Curtis *et al*, 1984). Finally the actual behavioural repertoire which allows the newly formed adult insect to

emerge from its old cuticle is dependent on the low, declining ecdysteroid titer. Eclosion hormone is only released and can only influence behaviour if the ecdysteroid titer declines (Truman, 1981). Both the magnitude and temporal pattern of the ecdysteroid pulse are exploited by the cells of the insect to time the cellular events necessary for moulting. Not only increases in titer are important but also there is a requirement for declining titers for the successful timing of some cellular and behavioural events.

Juvenile Hormone: Its Interaction with Ecdysteroid During the Moult Cycle

Juvenile Hormones

In 1934 Wigglesworth demonstrated that the metamorphosis of Rhodnius could be blocked by the administration of haemolymph from juveniles (Wigglesworth, 1934). The blood-borne factors action was essentially to juvenilise the recipient, hence it was termed juvenile hormone (JH). Since its discovery the chemical characterisation of JH has established it to be a sesquiterpene. Several hormonal species are known and JH0, JH1, JH2 and JH3 have now been isolated from the tissues of a variety of insect species (see Fig 1.5). At present the physiological significance of this diversity is not understood. It is possible that the different molecular forms of JH may have differing functions (eg, Lanzrein et al, 1975). Several good reviews are available (Gilbert et al, 1980; Riddiford, 1980c). In the presence of JH at the time of a moult larval characters are expressed, the titer of the JH determining the type of new cuticle formed (see below). At the moult from pupa to adult there is no detectable JH present and a fully formed imago results. From Fig 1.2

it can be seen that low levels of JH are present in the pupal moult to prevent precocious production of adult characteristics, but the highest titers occur during larval-larval moults when only juvenile features are expressed. Topical application of JH at the larval-pupal moult can induce a supernumerary instar by mimicking the high JH titer which causes a larval-larval moult. Conversely if the source of JH (see below) is removed by neck ligation or allatectomy the absence of JH causes precocious metamorphosis. Thus elimination of JH prior to a larval-larval moult causes precocious pupation or the production of a larval-pupal intermediate. Allatectomy prior to the larval-pupal moult, however, does not result in a precocious adult, although some structures do express adultoid characters (Kiguchi and Riddiford, 1978). Anti-juvenile hormones compounds such as precocenes will mimic this in some species (for review see Bowers, 1985).

The site of secretion of JH is the corpus alatum (CA) (eg, Granger et al, 1982). Just as the timing of ecdysone release from the PG is highly regulated both in terms of titer and temporal release so too is JH. However the precise mechanisms involved in the control of titer is not well understood and although it is reasonable to assume that they are secretion, metabolism and excretion the relative importance of these factors is not known. Specific haemolymph binding proteins do occur in some species which protect the hormone from degradation by esterases (for reviews see Gilbert et al, 1980; Del Kort and Granger, 1981). JH determines the qualitative features of the cuticle during insect development with a succession of different cuticle types being expressed throughout the life history of an insect according to the JH titer. The cellular events of moulting have been discussed in detail elsewhere in this chapter and are under ecdysteroid control. The role of JH in these

cellular events will now be briefly considered.

Interaction of JH and Ecdysteroid During the Moult Cycle

Throughout larval life JH modulates the actions of ecdysteroid. Prior to the moult it is known to promote DNA synthesis and mitosis, but this does not seem to be a prerequisite for other cellular events. The change in commitment of Manduca epidermis is a particularly favourable system in which to study JH action since genes for pupal cuticle structures are not immediately expressed following exposure to ecdysteroid in the absence of JH, but must wait until the subsequent larger peak of ecdysteroid initiates the pupal moult. It is thus possible to examine the molecular events that accompany the change in commitment independently by using cultured epidermal fragments (Riddiford, 1980b; Riddiford, 1984). One of the first detectable effects of ecdysteroid in this system is to turn off the expression of the genes that encode the major protein products of the cells. If JH is present this inhibition of expression (probably due to the cessation of transcription) is temporary. If JH is absent, the change is permanent. The mRNAs for various larval-specific proteins disappear with differing time courses. This has been taken to indicate (Riddiford, 1984) that different concentrations of ecdysteroid are required to repress different subsets of larval genes. How JH exerts its influence on gene expression is not known. However in Drosophila KC cells cytoplasmic JH receptors have been detected (eg, Chang et al, 1980) which become localised in the nucleus on exposure to JH (Riddiford and Mitsui, 1978). It has been postulated that this JH-receptor complex could bind to the chromatin in a manner analogous to ecdysteroid-receptor complexes and could induce some type of conformational change in the chromatin so that the 20-HE complex

would induce larval protein transcription. It must be borne in mind that as yet there is no direct evidence of JH-receptor complexes binding to chromatin of target cells and this area of molecular interaction awaits further investigation.

If the fifth larval instar of Manduca is studied, JH levels are maintained up until day 2. The presence of JH may function to promote the growth of the imaginal discs but prevent them from differentiating prematurely (Oberlander and Silhacek, 1976). On day 2 the levels of JH esterase increase dramatically (eg, Cymbrowski et al, 1982) and the CA is inactivated (Bhaskaran et al, 1980). As a result, the JH titer falls to an undetectable level in the larval tissues (eg, Fain and Riddiford, 1975 - see also Fig 1.2). On day 3 there follows the commitment peak of ecdysteroid and signs of a transient apolysis can be observed (Riddiford and Curtis, 1978). The cells of the epidermis are now irrevocably destined to express 'pupal' genes and are no longer competent to produce larval proteins (for reviews see Riddiford 1980b; 1980c; 1984). It could well be that several ecdysteroid-induced proteins are required to achieve the switching off of larval specific genes; the low endogenous levels of these proteins has hampered their study.

It is only when the epidermal cells are exposed to the second ecdysteroid peak that the cells begin to synthesise the structural proteins of the pupal cuticle. It is likely that the presence of JH during this peak serves to prevent the adult gene set from becoming activated prematurely (Kiguchi and Riddiford, 1978). For a full discussion of cellular events and their hormonal control in Manduca epidermis see Riddiford (1985).

This discussion should have made it clear that there is a complex interrelationship between ecdysteroid moulting hormones and the juvenile hormones throughout the postembryonic development of

insects. Not only are the titers of the two hormones mutually interdependent, but their actions in their target tissues also interact. This complexity has relevance to the use of the so-called 'third-generation' pesticides (insect growth regulators) that are based on JH. It is no accident that the range of symptoms produced by these substances is very broad. It is also no coincidence that the majority of these compounds kill insects at the time of the moult. Developmental derangement leading to the formation of larval-pupal intermediates is not the only way in which these products kill insects (Slama et al, 1974). A good example is the ability of JH analogues to prevent moulting altogether in the cockroach Blattella germanica (Masner et al, 1976). Here JH acts to prevent ecdysone secretion. Much remains to be discovered about ecdystoid-JH interactions, both at the level of control of hormone titers and at the level of their cellular actions.

Rationale for this Study

The PG play a central role in the moult cycle of Manduca sexta (see previously) and it was therefore of interest to investigate the possible existence of as yet uncharacterised molecules that may be important in the control or function of these glands. The approach taken was to generate MAbs to crude PG homogenates and to look for Abs directed against surface and cytoplasmic Ags with a view to investigating their possible role in PG function. Cell surface markers are potentially very useful in the study of development of the PG and the distribution of the Ag in, eg, homologous tissues. Another potential application of cell specific surface markers would be for the targeting of cytotoxins to selectively delete the PG in vivo. This has obvious advantages over currently used invasive techniques and would be invaluable in establishing the role of the

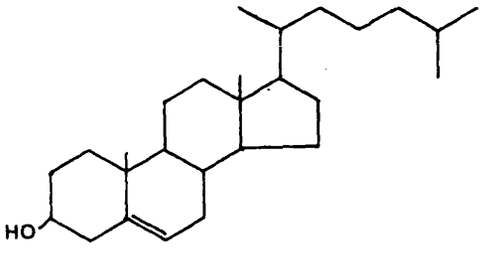
PG during development. In particular the function of the PG during embryonic development could be studied.

Also the possibility of developing a library of MAb to purified ecdysteroid was examined with a view to improving upon the specificity exhibited by conventional polyclonal antisera.

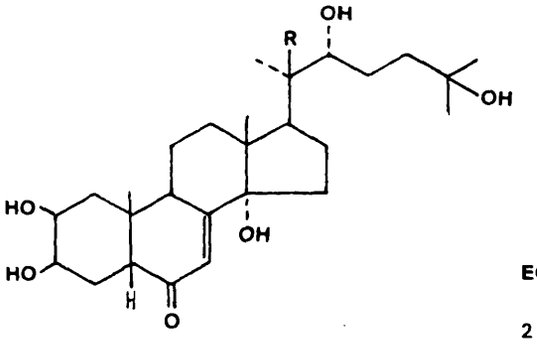
This thesis describes the production of MAb to 20-HE-BSA conjugates and the resulting profile of Abs generated and gives an indication of the most promising lines of approach for future studies.

There is also the identification of PG derived Ags, their occurrence during the insect's larval life, in particular the fifth instar, and an attempt at characterising and investigating the possible function for the Ags in the PG.

Fig 1.1 The Structures of Cholesterol, Ecdysone and 20-Hydroxyecdysone



CHOLESTEROL



ECDYSONE : R = H

20 – HYDROXYECDYSONE :

R = OH

Fig 1.2 Temporal Changes in Ecdysteroid and JH Titers During the Fourth and Fifth Larval Instars of Manduca sexta (after Riddiford, 1985)

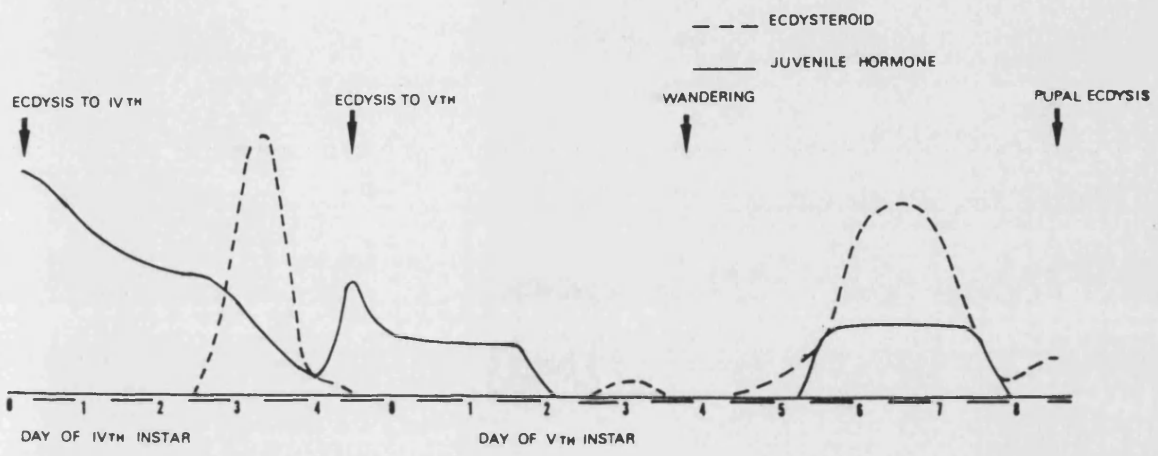

ECDYSTEROID
JUVENILE HORMONE
ECDYSIS TO IVTH
ECDYSIS TO VTH
WANDERING
PUPAL ECDYSIS
0 1 2 3 4 0 1 2 3 4 5 6 7 8
DAY OF IVTH INSTAR
DAY OF VTH INSTAR

Fig 1.3 Hypothetical scheme of the interaction between the fat body factor, JH, ecdysone and PTTH. The JH and ecdysteroid curves are after Riddiford (1985); the PTTH curves after Bollenbacher *et al* (1984); and the profile of FB factor activity derived from Watson *et al* (1984b)

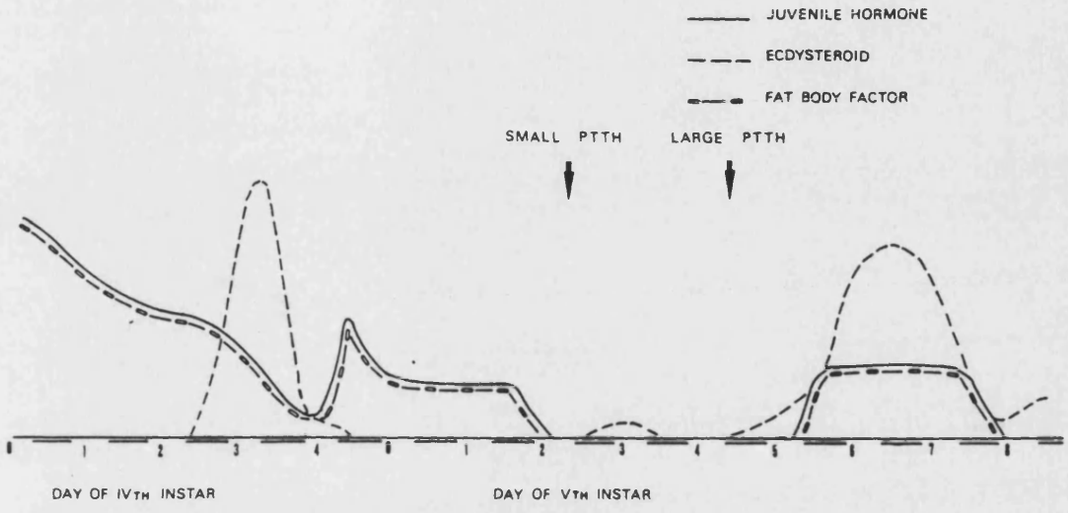
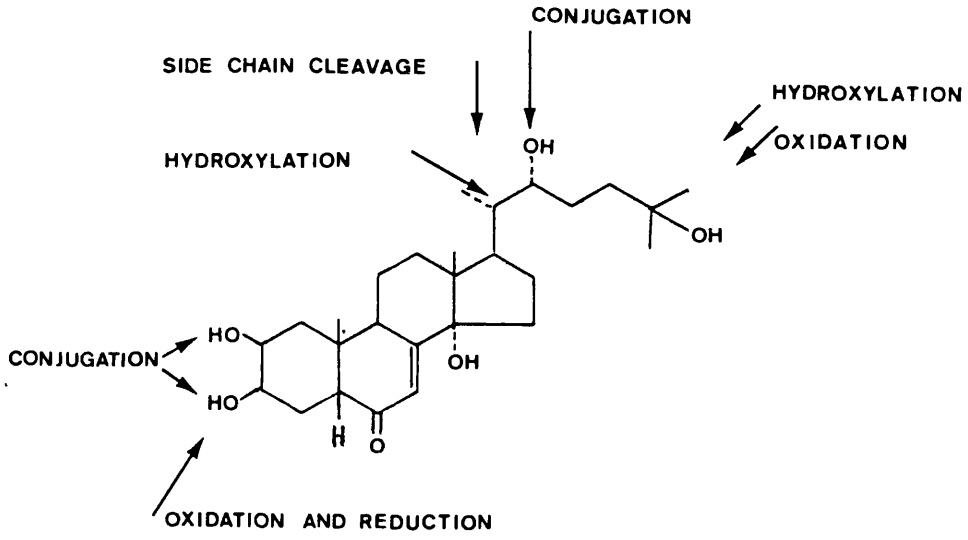
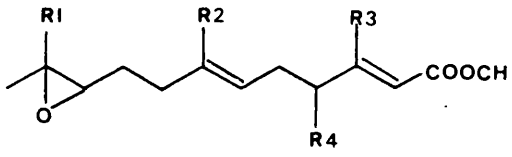

JUVENILE HORMONE
ECDYSTEROID
FAT BODY FACTOR
SMALL PTTH
LARGE PTTH
1 2 3 4 0 1 2 3 4 5 6 7 0
DAY OF IVTH INSTAR
DAY OF VTH INSTAR

Fig 1.4 Ecdysone metabolism: The reactions involved and the sites of reaction.

Fig 1.5 Structures of the Juvenile Hormones.





JH I	RI=C2H5	R2=C2H5	R3=CH3	R4=H
JH 2	RI=C2H5	R2=CH3	R3=CH3	R4=H
JH 3	RI=CH3	R2=CH3	R3=CH3	R4=H
JHO	RI=C2H5	R2=C2H5	R =C2H5	R4=H
ISO JHO	RI=C2H5	R2=C2H5	R =CH3	R4=CH3

Chapter II: Monoclonal Antibodies: Theoretical and Practical Considerations

The aim of this chapter is to provide a very brief overview of the vertebrate immune system and essential background to the experimental procedures used in this thesis. It gives reference to humoral defenses and provides insight into why antibodies have become such a powerful biological tool in both their polyclonal and monoclonal forms. Special attention is given to monoclonal antibodies and the techniques involved in their production.

Introduction

The immune system has evolved as a defense against infection. Invertebrates, such as the insects, have a cellular defense system along with various antibacterial proteins which are produced in response to infection. Although some specificity is exhibited by these proteins it is not clear whether they are differentially induced by different bacteria. This is an area of current interest, see for example Steiner et al (1981).

The vertebrate immune system has been subjected to much more rigorous study and a vast body of data is now available on how the vertebrate immune system functions. The vertebrates exhibit both a cellular and specific acquired immune response. For general discussions on the immune system see Golub, (1981); Roit, (1980) and McConnell et al (1981).

The two classes of vertebrate immune responses show distinct functional differences but act in concert to provide a highly effective and elaborately controlled defence system. Firstly, the

cellular class of response involves T-lymphocytes which mature under the influence of the thymus (Reinherz and Schlossman, 1980). The T-cells function as the major defense against intracellular organisms; they recognise antigen (Ag) by its interaction with specific receptors on the cell surface. Within the T-lymphocytes are functionally distinct subpopulations that are triggered differentially by Ag. The cytotoxic T-cells can directly kill foreign or virus-infected cells. The helper T-cells which can help B-cells to produce Ab also help other T-cells to produce cell mediated response as well as activate and recruit macrophages to the site of infection. The suppressor T-cells can actively inhibit B and T-cells and therefore have a regulatory role.

Macrophages are important cells in the cell mediated immune response as they present Ag on their cell surfaces which acts to trigger Ag-sensitive lymphocytes that perform the effector functions.

As previously mentioned T-cells also interact with the cells responsible for the specific acquired immune response, that is, the bone marrow-derived B-cells. These helper T-cells trigger B-cells to secrete specific antibody (Ab). A B-cell that encounters the Ag to which it is specific will not respond by secreting Ab unless it is also stimulated by a T-cell that has been activiated by the Ag. Very few Ags are capable of eliciting an Ab response without T-cell help. Interaction between T and B-cells is therefore essential in the production of an effective defense against infectious agents.

The Specific Immune System

The production of specific Ab by B-cells in response to Ag shows a characteristic pattern depending on whether or not the Ag has been encountered previously or is novel to the immune system. When an

animal is first exposed to an Ag there is a lag phase of several days before specific Ab production can be detected. There is an exponential rise in circulating Ab which then slowly declines. This is termed the primary (1^{o}) response. If the same animal is subsequently re-challenged with the Ag months or even years later the Ab production shows a reduced lag phase and a heightened response. It is sustained for a longer period of time than the 1^{o} response. This is termed the secondary (2^{o}) response. The immune system therefore 'remembers' previous challenge with Ag and mounts a more effective defense the second time the Ag is encountered. Humoral defense characteristically exhibits specificity. Each B-cell has the capacity to synthesise and secrete a single specific antibody molecule which is unique in its structure (Burnet, 1959). The diversity of Ab structure seen in the immune response is generated by a complex system of genetic rearrangement and somatic mutations in the B-cells' DNA (Tonegawa et al, 1981; reviewed by Leder, 1982). The antibody molecule that an individual B-cell will secrete is expressed as a cell surface receptor (Wigzell and Makela, 1970). If this receptor encounters the Ag to which it is specific the resulting Ag-Ab binding will trigger the cell to divide. The progeny of these divisions form a clone of cells all secreting Ab with identical specificity. Within this clone of cells are two functionally discreet subpopulations derived from the virgin B-cell when it first encountered the specific Ag. The first subpopulation is the memory cells which are long lived and continually circulate between the vascular and lymphatic systems. They are capable of responding more readily to Ag than the virgin B-cell. The other subpopulation is the effector cells that synthesise and secrete Ab that is characteristic of the 1^{o} response. On re-challenge with

Ag the memory cells produce the Ab that constitutes the 2^{o} response.

A prerequisite of such a defense system is that the host animal can distinguish its own molecules from those of foreign origin. In the immature animal the immune system becomes tolerant to self components by either eliminating B-cells that are specific to these self molecules or by actively supressing these B-cells by a subpopulation of T-cells known as T-supressor cells.

Biological Applications of Antibody

Abs can potentially be generated against an unlimited number of different Ags and are therefore of enormous importance as tools for the study and characterisation of molecules of biological interest. For this reason serum Abs have been raised against a broad spectrum of Ags for use as probes and in assay systems, etc. Many have been directed against hormones, eg, protein hormones such as human placental lactogen (Bosch *et al*, 1975) and 2-macroglobulin and 1-glycoprotein (Grenner, 1978) for detection and quantification of the hormones from biological samples. Similarly, steroid hormones have been used as the Ag against which serum Abs have been raised, eg, progesterone and deoxycorticosterone (Erlanger *et al*, 1959), testosterone, cortisol and aldosterone (Neri *et al*, 1964) and 17 -estradiol (Ferin *et al*, 1968). In some instances the purpose of raising the antiserum was to develop assay systems, in others to block the effects of endogenous hormone with the Ab. Serum components have also been effectively used as immunogen, ie, ferritin (Boenisch, 1976) and of course immunoglobulins themselves. This is not intended to be an exhaustive resume of the application of Abs to biological molecule detection and investigation, it serves

only as an indication of some areas of interest.

Antibody Production

Some of the factors that are important in Ab production will now be considered.

Immunoglobulin Classes

Ab molecules or immunoglobulins (Igs) are proteins that occur in the higher vertebrates in five main classes, IgA, IgD, IgE, IgG and IgM. Each has a different class of heavy chain (see Fig 2.1) which are α, δ, ε, γ and μ respectively. Each Ig class has its own functional role in the humoral defense system. A schematic diagram of the basic Ig structure is given in Fig 2.1. All Ig molecules share the same basic four peptide structure having two identical heavy and two identical light chains that are linked by di-sulphide bonds.

The most abundant Ig class in serum is IgG which has a mwt of 150KD and is characteristically produced during a 2^{o} immune response. In response to, for example, an infection by a micro-organism the specific IgG Abs will coat the invading organism and the exposed FC region of these Abs will then be bound by specific receptors on phagocytic cells. In this way IgG serves to enhance phagocytosis of foreign organisms. IgG FC regions can also bind to the first component of the complement cascade and this results in the activiation of this system which causes the destruction of micro-organisms by biochemical attack.

IgM Abs have a mwt of 900KD and are produced during the initial stages of the 1^{o} response and on secretion form a pentamer bearing 10 Ag binding sites. IgM is a potent activator of the complement

system and also agglutinates bacteria. During B-cell development IgM is the first class of Ig produced.

IgD is the Ig class that is found expressed on the cell surface of resting B-cells. Very few B-cells ever actively secrete IgD and it seems to function solely as a cell surface receptor.

IgA is the Ig found in mucous secretions and acts as part of the defense system of external body surfaces. It occurs as a monomer or a dimer.

IgE binds to mast cells and when cross linked by Ag causes the cell to degranulate and release histamine and various inflammatory mediators. This also leads to the local recruitment of granulocytes, polymorphs and eosinophils and acts as a second line of defense after IgA in mucous secretions.

In the production of a serum Ab the Ig subclass that will be induced depends on whether the response is 1^{o} or 2^{o}, IgM being produced early on in the 1^{o} response whilst IgG is predominantly associated with the 2^{o} response. The 2^{o} response has a higher titer of Ab than the 1^{o} and also often exhibits a higher affinity for the Ag because IgG Abs often bind the Ag with higher affinity than do IgM Abs.

Antigenicity

How potent a particular Ag is at inducing specific Ab production can be due to several factors. The mwt of an Ag determines whether or not it will be antigenic. Molecules of low mwt ($<$3KD) require linkage to a large carrier protein to render them antigenic. These low mwt molecules are known as haptens. The reason for this lack of antigenicity is not understood although it probably is a reflection of the molecules relatively simple structure or that they are easily

degraded by the host. Such haptens will however combine with specific Ab but cannot elicit its production unless linked to a carrier.

An Ag is usually composed of several sites to which Ab will be specific and these are known as antigenic determinants. The three dimensional molecular structure of the Ag contributes to the relative antigenicity of different parts of the molecule as some determinants will be more accessible to the immune system than others. The relative dominance of these antigenic determinants will be reflected in the spectrum of Abs directed against the molecule when an immune response is mounted.

Affinity

Serum Abs contain the product of many different Ab secreting B-cell clones, each recognising different antigenic determinants on the Ag and with differing strengths of association. Serum Abs are therefore termed polyclonal antibodies. It is the combined profile of all these Abs and the extent to which they bind the Ag that determines the characteristics of the polyclonal serum. Ab binding to an antigenic determinant is a reversible process, the forces being electrostatic, hydrogen bonds and Van der Waals. The extent to which dissociation occurs will determine the strength of binding. The degree of Ag-Ab binding is termed the affinity. For polyclonal Abs it is the combined affinity of each individual Ag-Ab interaction that is important and the term avidity is used to describe this association between multivalent Ags and the Abs that bind them. The more Ab molecules per multivalent Ag the less likely it is that the Ag-Ab complex will dissociate giving a higher avidity relative to the affinity of a univalent Ag. This effect varies depending on the affinity of the individual Ag-Ab interaction.

Titer

The titer of an Ab is a measure of both the affinity (or avidity) and the concentration of the Ab in the serum.

Polyclonal Antibody Production

The majority of polyclonal Abs that have been raised for use as biological tools have used rabbits or goats as the host animal. Typically a 1^{o} immunisation of Ag is given followed by a 'rest' period of one to two months, after which a 2^{o} immunisation is given on the premise that a 2^{o} immune response will result. (For review see Playfair et al, 1974). By repeated assay of serum samples the titer of the antiserum can be monitored and once it has reached a level high enough for the purpose for which it is intended the animal can be bled, the serum expressed and the resulting antiserum can then be characterised to determine its specificity.

Restrictions and Disadvantages of Polyclonal Antibodies

Some of the major limitations of polyclonal antisera will be listed briefly.

1. Specific antibodies can only be raised effectively to purified Ag.

2. The quantity of Ag required to elicit an Ab response can often be relatively large.

3. Different bleeds from primed animals can exhibit different characteristics in terms of specificity and titer because of the temporal changes associated with the 2^{o} immune response.

4. There is no homogeneity in supply of polyclonal Abs.

5. If an antigenic site on the Ag molecule is dominant it is often not possible to raise good antisera directed against lesser

determinates on the same molecule unless affinity purification is performed. Similarly if an immunodominant contaminant is present in the immunogen poor quality antisera will result as the response will not be fully directed against the desired Ag.

Monoclonal Antibodies

The polyclonal response to an Ag consists of many hundreds of individual B-cells each secreting homogeneous Ab but with different specificities and affinities. It would be highly advantageous if individual B-cell clones could be propagated to provide a source of monospecific, monoclonal antibody (MAb).

However, B-lymphocytes have a finite lifespan both in the host animal and under *in vitro* culture conditions because they are terminally differentiated. This seemingly insurmountable problem in the potential production of MAbs was solved by the now classical work of Kohler and Milstein (1975). They described how immortality could be conferred on individual B-lymphocytes by the fusion of spleen cells with myeloma cells.

It has been known for some time that myeloma cell lines would secrete MAb in culture (see review by Potter, 1972) but obviously myelomas are a limited source of MAb. However, if the properties of the myeloma cell could be conferred onto the normal, untransformed, B-cell then a hybridoma would result with the potential for unchecked cell division but with the power to secrete monospecific Ab.

The methodology described by Kohler and Milstein (1975) was relatively simple. A mouse was immunised with Ag and four days later the spleen cells removed and brought into close contact with myeloma cells in the presence of sendai virus so that cell-cell

fusion would occur. The fused cells contained both parental nuclei and so were heterokaryons; with time the nuclei fused to form a hybrid. These hybridomas were selected for and screened for specific Ab production. A percentage of the cells had the specificity of Ab determined by the parental spleen cell but in quantities attributable to a myeloma. Since MAb production was first reported (Kohler and Milstein, 1975) somatic cell hybridisation of this type has been extensively used and MAbs are now available to a large range of Ags of biological interest (see reviews by Melchers et al, 1978; Kennet, 1981; and Eisenbarth 1981). Some of the major advantages MAb exhibit by comparison with polyclonal antisera will be briefly listed.

1. MAbs can be raised against an immunogen which contains a complex mixture of molecules in addition to the Ag of interest. The resulting MAbs can be used to purify this Ag from, eg, crude tissue extracts.

2. Relatively low doses of Ag are required for immunisation by comparison to polyclonal Ab production, particularly if in vitro immunisation is used (see review by Reading, 1982).

3. Homogeneous Ab is produced exhibiting monospecificity.

4. Abs can be isolated that are directed against minor antigenic determinants of a molecule even in the presence of immunodominant antigenic sites.

5. A homogenous supply of Ab is available in potentially unlimited quantity.

However it should be recognised that MAb do exhibit some unusual properties by comparison with polyclonal Abs. If the Ag does not consist of multiple identical sub units (which is usually the case) the precipitation reactions attributable to polyclonal antisera are

not possible with MAbs because a three dimensional lattice is not formed (Milstein et al, 1980). Negative results with a MAb do not necessarily indicate that the Ag is not present but could simply be a reflection of the condition under which Ag:Ab binding is being measured. Only a slight conformational change of the antigenic determinant is required to prevent the monospecific MAb from binding. This leads to the phenomenon of assay specificity and can limit how useful a particular MAb might be (Haaijman et al, 1984). Antigenic determinants can be expressed by different molecular species either because of chance conformational similarity or can be homologous in terms of their evolutionary origin. Care must therefore be taken in interpreting cross reactivity. Finally, the properties of individual Abs are masked in polyclonal antisera because the average affinity and specificity is seen. However, MAb can exhibit any one of these properties because only a single Ab molecule is being isolated and so a range of specificity and affinity characteristics can be anticipated in the products of hybridomas.

Monoclonal Antibody Production

The aim of this section is to provide the theoretical considerations that underlie the production of MAb and then to give practical details of the protocols used in this project. There are several good reviews on practical aspects of MAb production (St. Groth and Schneidegger 1980; Goding, 1980; Kennet, 1981; Galfre and Milstein, 1981; Zola and Brooks, 1982).

Principles of MAb Production: Selection for hybridomas

The spleen cell from an Ag-primed mouse are brought into contact

with myeloma cells in the presence of sendai virus, or more commonly, polyethylene glycol (PEG), to promote cell fusion. (For discussion on mechanisms of cell fusion see Knutton and Pasternak, 1979).

The efficiency of fusion is relatively low. Because myeloma cells will grow indefinitely in culture a selection step is required that ensures that only hybridoma cells survive. To achieve this a myeloma cell line deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) is used. This myeloma line is incapable of incorporating hypoxanthine into DNA. There are two pathways by which DNA can be synthesised (a) by salvaging pre-formed bases from exogenous or endogenous sources, ie the salvage pathway or (b) by synthesising DNA from its basic components, ie 'de novo' synthesis (see Fig 2.2). Because it is X-linked there is only a single copy of the HGPRTase gene and so it was possible to select for cells that spontaneously arose deficient in this gene. It is these deficient cells that are chosen for use in spleen cell fusions because they can only utilise the 'de novo' pathway for ribonucleotide production. If the myeloma cells are grown up in the presence of hypoxanthine, aminopterin and thymidine (HAT selective medium; Littlefield, 1964) they will die. Aminopterin blocks the co-enzyme folic acid reductase essential for 'de novo' synthesis - both pathways cease to function and cell death results. This is the fate of any unfused myeloma cells. Unfused spleen cells are terminally differentiated and so die after a few days in culture. Therefore if the hybridomas are cultured in HAT selective medium they will live because the spleen cell parent acts as a source of HGPRTase and in the presence of hypoxanthine and thymidine the salvage pathway can be utilised to form DNA. The parental myeloma cell confers immortality on the hybridoma.

Fusion of Spleen and Myeloma Cells

The fusion typically involves spleen and myeloma cells in the ratio of 10:1 respectively. The cells are centrifuged to bring them into close contact and in the presence of PEG membrane fusion occurs. At this stage the cells are very susceptible to permanent membrane damage and may die as a result. The timing of exposure of the cells to PEG and the subsequent dilution of the PEG must be controlled very precisely. The cells are then transferred to *in vitro* culture conditions in the presence of HAT selective medium. As selection commences mortality is high. Typically within about two to five weeks (depending on the parental myeloma line used) colonies of hybridomas are visible growing in each well. Once these cells are confluent the culture supernatants can be assayed for specific Ab secretion. A variety of assay techniques can be used, eg, indirect immunofluorescence (Brooks *et al*, 1980), bioassay (Luben *et al*, 1979) enzyme linked immunosorbent assay (ELISA); (Pardue *et al*, 1983). Colonies found to be secreting Ab of the desired specificity can be expanded and then cloned in an attempt to ensure stability of the cell line. Bulk MAb production can be undertaken either by large volume cell culture *in vitro* or by ascitic fluid production.

The basic principles of MAb production are represented schematically in Fig 2.3.

Myeloma Cell Lines Available

Table 2.1 depicts the range of myeloma cell lines available for MAb production and outlines their status in terms of Ab secretion. The majority of the BALB/c mouse cell lines were derived from the P3/X63-Ag8 cell line which had previously been derived from a

myeloma cell line MOPC 21. This original cell line had been induced by intra-peritoneal (IP) pristane injection and the cell line secreted IgG1. Non-secreting variants were selected from this cell line. Obviously a myeloma line that secretes Ab will be disadvantageous as the Ab secreted by the hybridoma will contain this unwanted protein. If a myeloma cell line that produces Ig light chains is used only one in four Ab molecules secreted by the hybridomas will be fully coded for by the parental spleen cell. Therefore it is advisable to use a non-secreting myeloma line for fusions such as the P3/X63-Ag8.653 cell line.

Myeloma Cell Culture

For MAb production in this thesis the myeloma cell line P3/X63-Ag8.653 was used (see Table 2.1). It was derived from a BALB/c mouse and was developed by Kearney et al, (1979) from a subclone of the secreting myeloma cell line P3/X63-Ag8.

Myeloma cells were maintained in culture through approximately thirty consecutive passages. Personal experience has shown that cells lose their capacity to fuse successfully with spleen cells if they were cultured continuously for long periods of time or if they grow above a cell density of approximately 1 x 10^6 per ml. Cells were therefore periodically thawed from stocks maintained frozen under liquid nitrogen.

The myeloma cells were cultured at a density of approximately 5 x 10^5 cells per ml by periodic splitting of the culture, usually every 48 hours, and replacement of two thirds of the cell suspension with fresh medium. The cells were maintained in 25ml flat culture flasks (Nunc) in a 10 ml volume of medium. Prior to manipulation the cells were gently sucked into a pasteur pipette and expelled

repeatedly to flush the myeloma cells off the plastic upon which they form an adherent layer.

Feeder Cells

Considerations:- The purpose of feeder cells is to enhance culture conditions by supplying uncharacterised growth factors that are lacking in the culture medium. Fusions performed and plated out in the absence of feeder cells do show hybridoma growth. However, if feeder cells are present many more hybridoma colonies will grow successfully. The feeder cells may act to buffer the cells from sub-optimal conditions. These observations are supported by the work of St. Groth and Scheidegger, (1980) who observed variable degrees of success in the absence of feeder cells but obtained consistently good hybridoma growth in their presence. Inclusion of feeder cells is therefore probably a good practice although it no doubt depends on the fusion frequency and the parental myeloma cell line used.

Choice of feeder cell type:- There are several potential sources of feeder cells, some of the most commonly used types will be briefly discussed in this section.

Zola and Brooks (1982) used a suspension of spleen cells derived from unimmunised mice. Thymocytes have also been successfully used (Oi and Herzenberg, 1980), whilst Brodowski et al, (1979) used irradiated human fibroblasts. All these cell types were reported to enhance hybridoma growth.

Throughout this study peritoneal macrophages were used. Hybridoma colony growth is promoted when these cells are present and in addition they serve to clear much of the cellular debris that accumulates during the first few days post fusion. The other advantage of peritoneal macrophages is that they are easy and quick

to prepare and stocks can be maintained frozen under liquid nitrogen. It would of course be beneficial if cell-conditioned medium was commercially available to obviate the requirement for feeder cells.

Peritoneal Exudate Cell Preparation

BALB/c mice were used of both sexes and any age greater than eight weeks. A typical mouse was found to yield about 5 x 10^6 peritoneal exudate cells of which about 50% were lymphocytes. An appropriate number of mice were killed by cervical dislocation and submerged in 70% ethanol for 1-2 mins to surface sterilise the animals.

The mice were asceptically dissected, the skin being cut back mid-ventrally to reveal the intact peritoneum. 4-5 ml of sterile phosphate buffered saline (PBS) (Flow) was injected into the peritoneum close to the liver using a 26G needle until distension was observed. The abdomen was then gently massaged to facilitate cell detachment from the organs and peritoneal membrane. The PBS was then gently removed from the abdomen through a small incision in the peritoneum and was transferred by pasteur pipette to a 20ml sterile sample bottle. The exudate cell suspension was opaque. (Any discoloured cell suspensions were discarded in case the gut had been inadvertently ruptured). The cells were irradiated with 2000 rads from a ^{60}Co source (4.5 mins at 450 rads/min). (This prevented cell division by fibroblasts which have the potential to overgrow the hybridoma colonies.) The suspension of cells was spun at 200g for 10 mins and the supernatant discarded. The pellet was loosened by tapping and a small volume of complete medium (1-2ml) was added. The cells were counted and the volume adjusted to 4 x 10^5 cells per ml. For the preparation of fusion plates 50 ul

of the cell suspension was aliquotted into each well of a flat bottomed 96 well plate (Flow). The plates were incubated for between one and five days prior to the addition of the cells resulting from the fusion.

Selective Medium

Hypoxanthine and thymidine solution (HT) was available as a 50 x stock as was the hypoxanthine, aminopterin and thymidine solution (HAT) (GIBCO). The final molarities of the three constituents were as follows when made up in culture medium to give a 1 x solution. Hypoxanthine, 0.1mM., aminopterin, 0.0004mM and thymidine 0.016mM.

2ml of the 50x stock solutions were used to supplement 100ml of complete RPMI. Following the fusion HAT selective medium was used for fourteen days. Then HT supplemented medium was used for seven days so that the cells could eliminate the intracellular aminopterin prior to culture in complete RPMI.

Polythylene Glycol

PEG (4000) (Merck) was used as the agent to promote cell fusion. A 45% PEG solution in PBS was made up by adding 3.6g PEG to 4 ml PBS plus 400ul dimethylsulphoxide (DMSO). The PEG was autoclaved and stored at room temperature. The DMSO was included to act as a stabiliser to help protect the cell membranes from damage post fusion when they were most fragile.

The Production and Maintenance of Hybridoma Cells

There are four main stages in the production of Ab-secreting hybridoma cell lines (see Fig 2.2). First there is the fusion between spleen cells from a primed mouse and myeloma cells from an

established myeloma cell line. Second, there is the selection step in which non-fused myeloma and spleen cells are eliminated. Third, the population of growing hybridomas are screened to detect which are secreting specific Ab directed against the immunising Ag. Finally, the secreting cells must be cloned to select for stable MAb-secreting cell lines.

Fusion of Primed B-Lymphocyte with P3/X63-AG8.653 Myelomas

Spleen Cell Preparation

Four days after the final immunisation the primed BALB/c mouse was killed by cervical dislocation and submerged in 70% ethanol for 1-2 mins. The spleen was removed asceptically and transferred to a petri dish containing 10 ml sterile PBS. The PBS was taken up into a sterile 10 ml syringe and using a 26G needle the buffer solution was gently injected into the spleen. The spleen cells were gradually flushed out into the petri dish in the form of a suspension, once fully perfused the spleen appeared pale and membranous. The cell suspension was transferred to a siliconised 50 ml sterile glass centrifuge tube leaving any tissue debris in the petri dish. The cells were counted and the volume of spleen cells adjusted to give a total of 1×10^8 cells. (Any surplus cells were frozen under liquid nitrogen for potential use in subsequent fusions.)

Myeloma Cell Preparation

For successful fusion the myeloma cells must be in the exponential phase of growth with a viability of greater than 95%. Cells were cultured as previously described and split when confluent 48 hours prior to the fusion. Twentyfour hours prior to the fusion

the cells were spun down (200g, 10 mins) and resuspended in fresh complete RPMI. This yielded approximately 1 x 10^6 cells per ml with a viability of 95-98% on the day of fusion. 10 ml of this cell suspension was fused with the 1 x 10^8 spleen cells.

Fusion

10^8 spleen cells and 10^7 myeloma cells were mixed together in a 50 ml siliconised glass centrifuge tube and the volume made up to 45 ml with PBS. The cells were spun at 200g for 10 mins and the supernatant discarded and the pellet loosened by tapping the tube. Whilst continuously shaking the tube by hand the PEG solution was added dropwise with the addition of 1 ml over 60 secs. The tube was then agitated in a water bath at 37°C for 90 secs. PBS was gently added dropwise whilst continuously shaking to prevent cell aggregation. Over the first 30 secs 1 ml of PBS was added, over the next 30 secs 3 ml and then gradually increasing the rate until a volume of about 25 ml was reached. At this stage the shaking was stopped and the centrifuge tube filled with PBS by decanting. The cells were spun at 200 g for 10 mins and the supernatant discarded. The pellet was loosened in 2 ml HAT selective medium and transferred to a sterile bottle containing 98 ml HAT medium. The pellet was allowed to break up over several mins. The cell suspension was mixed gently and 200 ul pipetted into each of 480 wells (5 x 96 well plates (Flow) that had been previously prepared with macrophage feeder cells). The plates were incubated. Portions of the fused cell suspension were often frozen down at this stage to reduce the number of wells to be screened at any one time (Harwell et al, 1984).

Feeding of Hybridoma Cells

The time course described is for P3/X63-AG8.653 derived

hybridomas under the conditions stated.

Two days post fusion the majority of cells in the wells had died because the unfused myeloma and spleen cells had been selected against. By day 4 or 5 a few large round cells with large nuclei could be seen and were showing signs of cell division and small colony formation. These cells were the hybridomas and by day 6-10 approximately 5 to 10 colonies were present per well in a typical fusion. By day 6 the cells required feeding with fresh culture medium. 200 ul of HAT medium in the wells was carefully removed using a multichannel pipette (Flow) and replaced with fresh HAT medium. Cells were fed in this way every 48 hours. By day 14 the wells were ready to assay. The HAT medium was replaced with HT for one week and then complete RPMI was used. Cells were fed daily once confluent. Typical fusion frequencies observed (that is the percentage of wells showing hybridoma growth) ranged from 80-95% during the course of this study.

Screening

This is discussed in detail in the relevant chapters. It is worth mentioning that it is essential that the screen reflects the ultimate application of the Ab once produced and that the assay system is fully established prior to doing the fusion.

Expansion of Cells of Interest

Wells that contained hybridomas secreting Abs of interest will be termed positive wells.

Cells from positive wells were expanded into larger volume (2.5 ml) wells in 4 well plates (Nunc) ie, from a volume of approximately 200 ul to a volume of about 1.5 ml. The wells were prepared with macrophage feeder cells prior to this expansion. Once the cells

were confluent some of the cells were frozen down and stored under liquid nitrogen whilst the remaining cells in culture were cloned to establish stable, Ab-secreting cell lines.

The following section will discuss the major cloning techniques available and detail the method of cloning used throughout this study.

Cloning

Positive wells are likely to contain hybridoma cells producing heterogeneous Ab. The wells may also contain cells that are not synthesising and secreting Ab. These cells probably have a more rapid generation time because a greater proportion of resources are directed towards growth.

To ensure homogeneous Ab and to prevent overgrowth of the cells of interest by non-secreting cells, the hybridomas must be cloned. A single cell is isolated and allowed to divide and form a clone of identical cells. The supernatants from such clones can be assayed and in this way cell lines selected that are producing MAb of interest.

The two most commonly cited cloning methods are cloning in soft agar and cloning by limiting dilution.

Cloning in soft agar is generally less efficient than limiting dilution because a higher cell density is required, ie 10^3 cells per ml as opposed to 0.5 - 5 cells per ml in limiting dilution. The procedure is as follows:-

Cells are diluted and immobilised in agar and as individual cells grow they form a tight clone of cells that becomes a colony visible by eye. This clone is then picked out and transferred into liquid culture. Because of the relatively high cell density

required for this technique there is a fairly high probability that two cells will have been in close enough proximity to form the colony. What appears to be a single colony could well be derived from two or more hybridomas.

Limiting dilution is the most frequently cited technique. Cells are diluted out in culture medium such that statistically there will be on average one cell per well. A percentage of wells will contain multiple cells and some will contain no cells and so repeated cloning is essential to ensure the cell line is truly clonal. This also applies to cloning in soft agar.

A disadvantage of both techniques is that the cells must be available at a relatively high cell density in order to be counted accurately. The risk is therefore run that non-secreting cells may overgrow the cells of interest before they can be cloned.

A slightly less conventional method of cloning was adopted throughout this study and has a number of advantages over conventional cloning methods. Hybridoma cells are individually placed in separate wells using a drawn pasteur pipette held in a micromanipulator. By the use of an inverted microscope cells can be individually selected, sucked into a reservoir of medium and transferred to wells with feeder cells. The advantage that this method offers is that cells can be cloned from a very low initial density, ie, from fusion wells prior to expansion. This reduces the chances of overgrowth by non-secreting cells considerably. Also, with practice, virtually all wells prepared contain only a single cell and several repeated cloning stages are therefore not required. In this study it was found that usually two successive clonings were sufficient. Additionally, instead of having to plate out hundreds of wells 50-100 wells are adequate in guaranteeing

successful perpetuation of a cell line. The technique is also very rapid once practised.

Irrespective of the cloning method adopted feeder cells are essential as very few cell lines will remain viable at a cell density of approximately 4 cells per ml.

Cloning by Micromanipulation

One 96 well plate (Flow) was used for each cell line to be cloned and was prepared with feeder cells 24 hours prior to cloning. The volume of complete RPMI per well was 200 ul. (If cells were cloned directly from fusion wells HAT or HT medium was used as appropriate). The inverted microscope and micromanipulator were placed in a sterile flow hood and a pasteur pipette drawn out to a fine tip and attached to the micromanipulator. The plugged end of the pasteur pipette was connected by plastic tubing to the mouth so that cell uptake could be finely controlled by suction. The pipette was gently filled to provide a reservoir of medium. Single healthy cells were selected, drawn into the pasteur and expelled into a well of the 96 well plate. This was repeated until 50-90 wells had been seeded with single cells. The cells were incubated.

After 6 days the cells were examined under the inverted microscope and only wells with single colonies were noted for subsequent assay on about day 14. After repeated cloning of the chosen clone all wells were secreting specific Ab and so the most positive well was selected for expansion for freezing and for bulk MAb production.

Stability of Hybridoma Cell Lines

At the time of fusion two diploid cells, each with 40

chromosomes, fuse to form a cell with a complement of 80 chromosomes. The nuclei do not fuse immediately but when a single nucleus is formed there is a state of instability and chromosomes are actively eliminated. This process may be random in mouse: mouse cell fusions but in mouse: human cell fusions the chromosomes derived from the human B-cell are preferentially eliminated relative to the chromosomes of the mouse myeloma. (Zola and Brooks, 1982).

If a chromosome is eliminated that is essential for, eg. cell division or cell growth, then the cell will die. If however a chromosome is lost that is required for Ab production the cell will continue to grow and divide, probably at a more rapid rate than Ab secreting cells, but it will not secrete Ab.

It is these cells that can at any time arise and dominate the culture leading to a gradual loss in the Ab secretion from a population of cells.

A state of true stability is never reached within the nucleus but the longer the cell remains stable the less likely it is to become a non-secretor. When selecting clones to re-clone or expand for Ab production it is always advisable to freeze down a backup cell line from the clone in case non-secreting cells arise within the primary clone.

Storage of Cell Lines

The lower the temperature at which cells are stored, the longer they will remain viable. Liquid nitrogen ($-196^{o}C$) is the most suitable method for long term storage.

The cryoprotective solution used had the composition (v/v) of 50% FCS, 20% DMSO, 30% complete RPMI. Cells were spun for 5 mins at 200g. The supernatant was discarded and 0.5 ml complete RPMI was

added to the cell pellet and the cells cooled on ice for 15 mins. 0.5ml of the cryoprotective solution (see above) was placed in a vial (Nunc) and cooled on ice for 15 mins. The cell suspension was gradually added to the cryoprotective solution and the vial was shaken and placed in a 'slow cooler' in the vapour above the liquid nitrogen. Alternatively the cells were placed in a -90°C freezer to initiate the cooling process. (The optimal rate of cooling is 1°C per min, the DMSO acts to prevent ice crystal formation that would otherwise disrupt the cell membranes).

After 24 hours cooling the vial was plunged into liquid nitrogen for long term storage.

From experience cells were found to maintain their viability at a temperature of -90°C for 8-10 weeks but 1-3 years under liquid nitrogen storage. Periodic thawing and sub culturing was practised to maintain viability for cell lines in long term storage. Cells were also re-cloned once every 6-12 months to ensure the maintenance of viable MAb-secreting cell lines.

Cell Recovery

The cells were thawed rapidly to 37°C either by placing them in a 37°C water bath or by the addition of pre-warmed culture medium. The cells were washed in three changes of medium by repeated centrifugation at 200g for 5 mins. The cells were then cultured in the presence of peritoneal macrophages.

Bulk Antibody Production

Two methods were used to produce large quantities of MAb from cloned hybridoma cells. The first was to culture cells and expand them into several 25 ml culture flasks. Culture supernatants were

harvested from flasks that had reached a density of approximately 1 x 10^6 cells per ml. This typically yielded 5-50 ug per ml Ig.

The second method was ascitic fluid production. Hybridomas were injected (IP) into mice to produce solid and liquid tumours that secreted Ab. This Ab-rich fluid was harvested and typically yielded 10-60 mg per ml Ig. The ascitic fluid production method was as follows:

For each cell line 6 BALB/c eight week old male mice were injected IP with 0.5 ml pristane (tetramethylpentadecane (Sigma)) two weeks prior to injection with the cell line. (The pristane served to promote tumour formation and is an oil which acts as an irritant on the peritoneal membrane and gut mesenteries.) After two weeks about 1 x 10^6 hybridoma cells were injected IP into each mouse and the mice were left for a further two weeks. Once the mice showed visible signs of abdominal swelling the ascitic fluid was 'tapped off' by the gentle insertion of a 19G sterile needle into the peritoneal cavity of the mouse. The fluid was dripped into a glass vial. This process of 'tapping' was continued for the remainder of the life span of the animal, usually around seven days. The yield of ascites varied with animal depending on whether solid or liquid tumours predominated. On any one day yields varied from as much as 5 ml to only 200 ul depending on the animal. It was observed that the Ab titer was usually low on the first two days when the ascites was assayed but increased rapidly after this initial period.

Ascitic Fluid Purification

The fluid was allowed to stand at room temperature for one hour to allow any fibrin clots to form. After centrifugation (Eppendorf

5412) for 5 mins the supernatants were removed and stored either frozen at -20°C, or at 4° with sodium azide (0.001% w/v) depending on the previously determined storage properties of the particular Ab. Once all the ascitic fluid had been accumulated the Ab was precipitated with ammonium sulphate.

Ammonium Sulphate Precipitation

The stored aliquots of crude ascitic fluid were thawed and warmed to room temperature. Solid ammonium sulphate was added to continuously stirring ascitic fluid at a concentration of 0.3 g per ml which gave 40% saturation. Once the ammonium sulphate had dissolved the ascites was stirred for 30 mins at 4°C. After spinning at 4000 rpm (Beckman J6) for 30 mins at 4°C the supernatant was removed and stored, being later assayed to estimate losses of Ab. The pellet was resuspended and dissolved in PBS (pH7.2, 0.15M) back to the original volume of ascites. The precipitation and centrifugation steps described above were repeated. The supernatant was again retained to estimate losses of Ab. The pellet was resuspended back to the original volume in PBS and this solution was dialysed in visking tubing (Medicell) against PBS for 48 hours at 4°C. The PBS was changed twice during the course of dialysis and gently stirred throughout. The Ab was recovered from the visking tubing and stored according to the stability of the Ab concerned.

Measurement of Ab Concentration in Precipitated Ascitic Fluid

Samples were spun in a microcentrifuge (Eppendorf 5412) for 5 mins and the supernatant retained for the analysis. In a quartz cell a sample of known dilution was placed in the u.v. beam of

spectrophotometer (Cecil) and the absorbance measured at 260 nm and 280 nm. The ratio of absorbance at 260:280 was then calculated:

Protein concentration = 1.55 x absorbance 280 nm - 0.77 x absorbance 260 nm (mg/ml)

(Allowance was made for the initial known dilution of the ascitic fluid).

Characterisation of MAb

The principles of characterisation will be discussed briefly in this chapter. For detailed specific examples of MAb characterisation see chapters III, IV and V.

Specificity

The first major consideration in the evaluation of how useful a given Ab will be is its specificity. This can be investigated by screening the MAb against Ags that can be considered to be potential cross reactants. How rigorous this needs to be depends on the ultimate application of the Ab. For example if it is to be used in an assay system to measure, eg. a hormone secreted by tissue *in vitro*, then providing no cross reactants are present in the system the fact that it may cross react with them is of minimal importance. However if the assay is to evaluate the hormone in serum samples where potential cross reactants may be present in the form of precursors and metabolites, then the specificity of the MAb is of the utmost importance.

The assessment of specificity is discussed in chapters where relevant.

Titre

Titre is a measure of the quantity of Ab present but also reflects the affinity of the MAb for the Ag. In practical terms this serves as an indication of how much Ab is required and this in turn depends on the application of the Ab.

This was measured using the screening assay and determined the highest dilution to give a positive result in a doubling dilution system. The titer was always re-assessed following MAb storage and MAb thawing.

Affinity

The binding kinetics of MAb:Ag are not usually investigated. For most purposes an empirical assessment is made as to whether or not the MAb show a sufficiently high affinity to be of practical use. However MAbs do provide useful tools for the investigation of Ag:Ab binding kinetics. (Mason et al, 1980).

In this study the affinity was not measured but assessed empirically.

Stability

This is essentially a practical consideration. A small portion of Ab should always be tested under the prepared storage conditions before committing the remainder of the Ab. Many MAb may be unstable under certain conditions (this is particularly true of IgM MAb) and it is often advisable to store such MAbs 1:1 (v/v) with glycerol at $-20^{o}C$. Repeated thawing and freezing will damage most Abs be they polyclonal or monoclonal in nature.

An aliquot of each Ab to be stored was tested by subjecting it to the proposed storage conditions and comparing the titer pre-and

post-storage. IgM MAbs were stored 1:1 (v/v) with glycerol at -20°C as they were more stable under these conditions than direct storage at -20°C.

Ig Class Determination

The Ig class of a MAb determines whether or not it can be used with eg, protein A, whether or not it will fix complement, etc. It is therefore useful to ascertain this characteristic of the MAb.

Ig class determination was made using an Ouchterlony diffusion kit (Serotec). In the central well 75 ul of the MAb was placed (either neat culture supernatant or diluted ascitic fluid). In the surrounding wells were placed 10 ul of each of specific Abs directed against the different mouse Ig isotypes ie, IgG1, IgG2a, IgG2b, IgA, IgM and IgE. The gel was humidified and left at room temperature for 24-48 hours. Precipitation bands could be visualised by eye between the central well and the well containing the anti-Ig class Ab which was directed against the same Ig class as the test Ab. Determinations, wherever possible, were performed on culture supernatants as ascitic fluid often contains host Ab that leads to the formation of multiple precipitation bands.

Monoclonality

There is no irrefutable way of demonstrating monoclonality of an Ab only that it is highly likely on the basis of good cloning practice and that it exhibits specificity.

In this study it was assumed to be the result of rigorous cloning practice.

Monoclonal Antibodies in the Study of Invertebrate Systems

MAbs have great potential for the study of developmental processes and physiological systems in both vertebrates and invertebrates. The majority of reports to date have applied MAb techniques to the investigation of vertebrate biology but this is undoubtedly a reflection of the relative work effort in the two fields and is not an indication of MAb applicability.

For the most part MAbs have been directed against cell surface Ags to identify phenotypic differences between populations of cells. Vertebrate studies have included investigations of such diverse cell types as neurones in the central nervous system (CNS) (eg, McKay and Hockfield, 1982; Hockfield and McKay, 1983) and pre-B cells and B cells of the vertebrate humoral immune system (eg, McKearn et al, 1984). There is a growing literature on tumour specific Ags (eg, Webb et al, 1983), and also the application of MAb directed toxins against specific tumour Ags for the treatment of some cancers (for review see Arnon and Sela, 1982; Poste and Kirsh, 1983). Success has also been reported in the production of MAb to human growth hormone (Ivanyi and Davies, 1980), to the calcium binding protein, calmodulin (Pardue et al, 1983) and to proteins from the rod outer segment of the frog (Witt et al, 1984). This study is worthy of a more detailed discussion because it combines the generation of MAb to proteins of known functional importance along with the use of MAb to probe for novel proteins of unknown status in the response to light in the retina. A protein extract from the frog retina was used as immunogen but also a second immunogen was prepared and used for a second fusion and consisted of soluble and peripheral membrane proteins. From the fusion using spleen cells primed with the protein extract many MAbs were isolated

which bound to rhodopsin, a protein which is important in the light response. MAbs were also isolated which had anti GTP-binding protein activity which is a protein important in the initial events within the retina which occur in response to light. However, two MAbs which were isolated from the second fusion were found to bind to previously unknown proteins the functional status of which is as yet unclear but by virtue of the MAbs can now be fully investigated.

The application of hybridoma techniques to the study of invertebrate systems has not been to such a diverse or extensive repertoire of Ags by comparison with vertebrate studies. Many have been used to distinguish subpopulations of neurones and one example is the study done on the leech, Haemopis marmorata (Zipser and McKay, 1981). A library of MAbs was raised against crude neuronal material. Interestingly one cell line secreted MAb that recognised four cells involved in sensory perception (pressure cells), the function of which was already known, but also identified other pairs of cells which are of unknown function but share a common surface determinant with the pressure cell neurones. This type of approach will be very useful in ultimately establishing the type of molecular mechanism that are involved in neuronal specificity.

Interest in the molecular genetics of neuronal development has led to some studies in which MAbs have been raised against cell surface Ags on Drosophila tissue. The extensive knowledge of Drosophila genetics and molecular genetics should be very advantageous in establishing the control mechanism which operate during development. One such study is that of Fujita et al (1982). This study was concerned with the isolation of neurones related by virtue of MAb binding. Many of the MAbs generated against a crude neuronal tissue immunogen gave general tissue staining. One MAb

specifically stained nuclei and obviously recognised a highly conserved nuclear Ag in that the MAb also stained vertebrate, invertebrate and plant nuclei. Of the more selective MAbs some only stained regions of the head such as brain, retina and nerves. The most interesting staining patterns were observed with MAbs which showed specificity within the nervous system. One MAb stained nerve fibres located in the optic lobes and brain and, from other MAb staining patterns, it was clear that the brain lamina and medulla differ antigenically from the rest of the brain. Selective staining of photoreceptor cells was also exhibited by another MAb which acts as a useful probe for the study of developing photoreceptor cells in the eye imaginal disc which is attached to the larval brain. Apart from the initial characterisation of some of the Ags by immunoblots the study was preliminary with respect to relating any of the specific molecules to the molecular genetics of the species.

MAbs have also been used to study the modulation of neural Ag during metamorphosis of Drosophila melanogaster (White et al, 1983). Some Ags were found to persist throughout the fly's life irrespective of stage while others showed drastic alteration with respect to concentration in synaptic areas at the time of metamorphosis. These Abs should prove useful in establishing some of the molecular mechanisms involved in modifying the CNS during metamorphosis.

One of the best applications of hybridoma techniques to the study of the invertebrate nervous system has looked at the establishment of embryonic neuronal pathways connecting the periphery and the CNS in the grasshopper (Ho and Goodman, 1982).

The MAb in question stains the pairs of pioneer neurones in the limb buds and antennae which had previously been demonstrated to establish the first pathways that connect the periphery to the CNS (Bate, 1976; Keshishian, 1980). However the MAb also stained an array of peripheral neurones responsible for establishing the first pathways which, prior to this study, had not been functionally defined. In this way, by looking for MAbs that recognise functionally discrete neurones and then examining other cells recognised by the same MAb some of the early events in the establishing of a peripheral nervous system have been elucidated. This study is now being extended to examine the factors that influence the peripheral growth cones and the extent to which cell-cell interactions are responsible for the pathways that are established. More recently Kotrla and Goodman (1984) have reported the application of MAb to the study of neurones and muscle pioneers that have a common pathway and all share a common target muscle. These types of approach are invaluable in the study of events in the development of an insect nervous system because they are able to shed light on functional relationships between the cell types involved and indicate the factors important in dictating the patterns of nervous connections which are laid down.

Other MAb studies directed against invertebrate derived Ags include the production of MAb against purified proteins for assaying levels of, ie, vitellogenin during the development of the mosquito, Aedes citropalpus (Ma et al, 1984) and purified steroid hormones (see chapter III of this thesis).

This is by no means an exhaustive resume of the work using MAb to study vertebrate and invertebrate systems but serves to indicate areas of interest and some of the applications of hydridoma technology.

Table 2.1 The Ab secreting properties of some commonly used myeloma cell lines.

Species/Strain	Myeloma Cell Line	Ab Secretion Status of Cell Line
Mouse/BALB/c	NSI-Ag4-1	Synthesises light chains but does not secrete them. Hybridomas can secrete mixed Ab molecules which contain myeloma light chains.
Mouse/BALB/c	P3/X63-Ag8.653	Does not synthesise or secrete Ig chains.
Mouse/BALB/c	SP2/0-17g14	Does not synthesise or secrete Ig chains.
Mouse/BALB/c	NSO	Does not synthesise or secrete Ig light chains.
RAT/Lou	Y3	Synthesises Ig light chains.

Fig 2.1 The Structure of Immunoglobulin

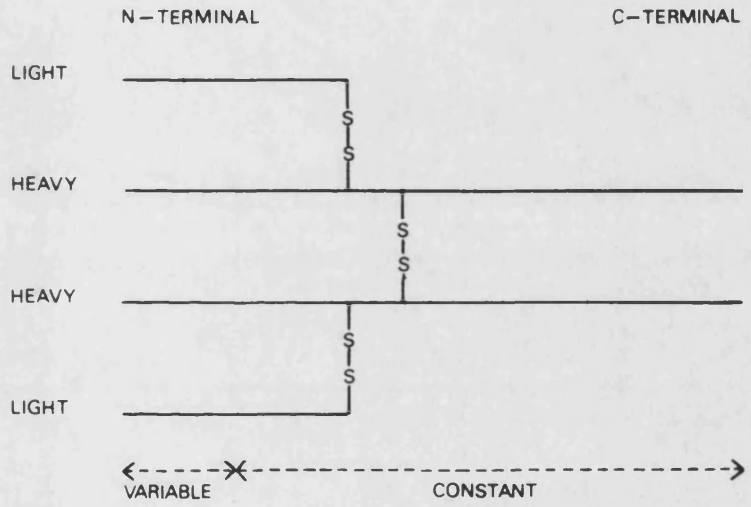

N – TERMINAL
C – TERMINAL
LIGHT
HEAVY
HEAVY
LIGHT
S
S
S
S
S
S
VARIABLE
CONSTANT

Fig 2.2 Schematic Representation of Hybridoma Selection

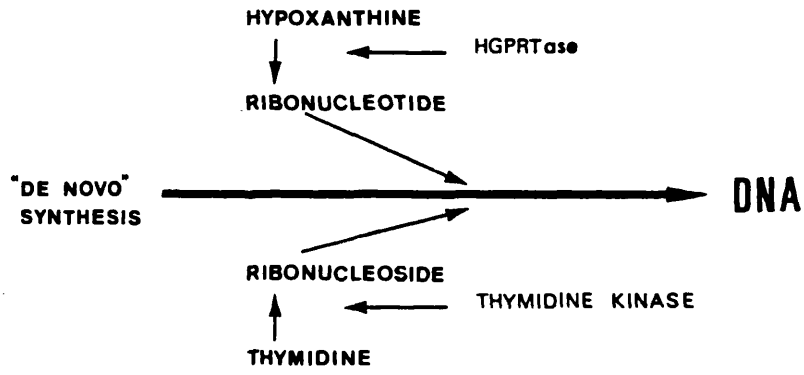
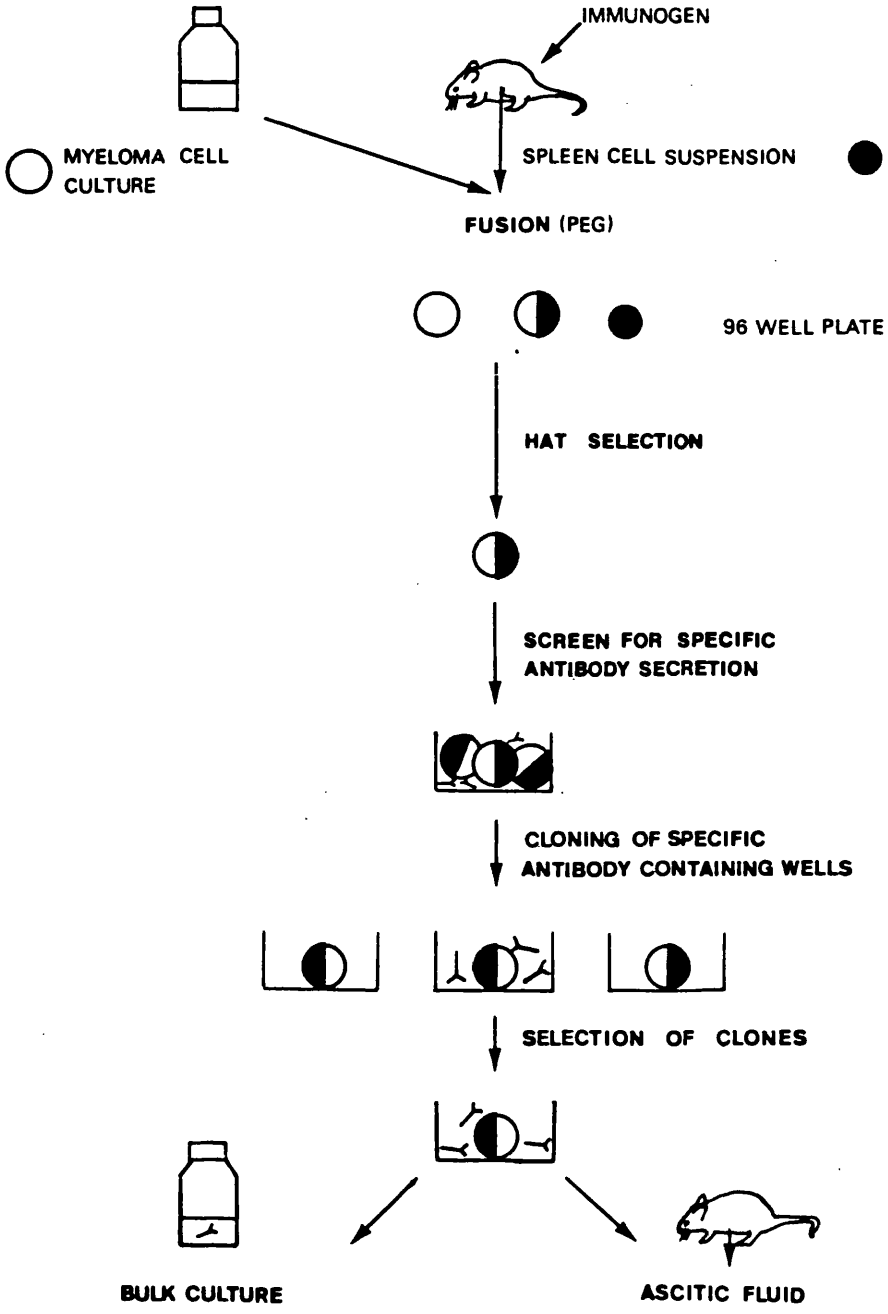

HYPOXANTHINE
HGPRTase
RIBONUCLEOTIDE
"DE NOVO"
SYNTHESIS
DNA
RIBONUCLEOSIDE
THYMIDINE KINASE
THYMIDINE

Fig 2.3 Schematic Representation of Monoclonal Antibody Production


IMMUNOGEN
MYELOMA CELL CULTURE
SPLEEN CELL SUSPENSION
FUSION (PEG)
96 WELL PLATE
HAT SELECTION
SCREEN FOR SPECIFIC ANTIBODY SECRETION
CLONING OF SPECIFIC ANTIBODY CONTAINING WELLS
SELECTION OF CLONES
BULK CULTURE
ASCITIC FLUID

Chapter III: Antibodies Directed Against 20-Hydroxyecdysone

Introduction

Abs directed against ecdysone and 20-hydroxyecdysone (20-HE) have been used successfully in RIA to measure ecdysteroid titers throughout the development of a variety of insect species (Lazarovici et al, 1983; Hirn and Delaage, 1980). Anti-ecdysteroid Abs in RIA have also been used in conjunction with thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), for example Zhu et al, (1983). To date the antisera raised have all been polyclonal in nature and have exhibited differential specificity for ecdysone and 20-HE (see Table 3.1). The Abs have also shown differing extents of cross reactivity towards ecdysteroid precursors and metabolites. Because of the complexity of these specificities the quantities of ecdysone measured by RIA are always expressed as 'ecdysone equivalents' or as '20-HE equivalents', according to the nature of the competing unlabelled ligand, as there is always a degree of uncertainty as to the exact nature of the molecules recognised by the antiserum, particularly in tissue samples. The characteristics of some of these antisera are summarised in Table 3.1. In all cases the immunogen was pure, synthetic ecdysteroid coupled to a protein carrier.

A more satisfactory method of quantifying particular ecdysteroids in tissue samples would be to have a library of MAbs that would discriminate between the different circulating ecdysteroids with a high degree of specificity. This would not only provide a more accurate estimation of ecdysteroid titer but would

also provide useful probes for the study of biosynthesis and metabolism of ecdysteroids. It could be envisaged that 'cocktails' of such MAbs could be made to provide the type of molecular discrimination required to investigate specific molecules of interest. This chapter is concerned with investigations to establish the feasibility of MAb production against ecdysteroids.

Antigenicity of Ecdysteroids

Like other low mwt molecules (approximately less than 3 KD), ecdysteroids are not innately antigenic. Whether this is because they are more rapidly degraded than higher mwt molecules or because they lack a complex structure is not known. Ecdysteroids can therefore be considered to be haptens (see chapter II) and require coupling to a large mwt carrier protein to render them antigenic (Odell et al, 1972). Despite their low mwt the hapten can combine with pre-formed Ab because it is recognised by the immune system as an antigenic determinant on the carrier protein. On initial exposure to a hapten-carrier T-lymphocytes become primed to the carrier protein and the B-cells respond to the antigenic determinants of the protein including the hapten. On second exposure with the hapten-protein complex the T-cells recognise the carrier and respond by providing 'help' to the hapten specific B-cells which are stimulated to divide and secrete Ab. Therefore it is only possible for successful anti-hapten Ab production to take place if T-cell help occurs and this is particularly true if the hapten is monovalent. This is because to elicit the response of a B-cell to Ag the Ag must cross-link surface Ab receptors and this requires repeated antigenic sites. In the absence of repeated

antigenic sites no cross linking occurs and so no response can be induced unless T-cell help is available.

Hapten-carrier Production

The earliest attempt to couple steroid to protein occurred in 1936 (Landsteiner and Van der Scheer, 1936) but the products lacked stability and successful conjugates were not produced until 1957 (Erlanger et al, 1957). (The failure of the initial attempt was probably due to the instability of the ester bond used to link the steroid to the protein).

To raise a specific antiserum the hapten configuration on the carrier must be constant so the steroid is derivatised at a single site and is then linked to the protein. Abs are usually directed against the part of the molecule distal to the site of coupling (Horn et al, 1976). Derivatives commonly formed include oximes, acylchlorides and hemisuccinates (Erlanger et al, 1957; 1959).

The protein can be coupled to the derivative by the mixed anhydride technique or by the carbodiimide method (Erlanger et al, 1957). The protein chosen to be the carrier is usually of relatively high mwt such as a serum albumin or thryoglobulin (see Table 3.1).

Having formed a stable protein-steroid conjugate the next consideration is how many steroid molecules have been coupled to each molecule of protein. This gives an indication of the antigenicity of the immunogen in terms of Ab response directed against the hapten. The methods of analysis of molar ratios vary. Porcheron et al (1976) measured the radioactivity of the conjugate having included a tritiated hormone marker in the derivatisation. Erlanger et al (1957) used u.v. absorbance analysis which measures

the contribution the steroid moiety makes to the combined absorbance of the hapten carrier in the final conjugate. Having measured the molar ratio, the antigenicity of the conjugate can be deduced; Nieshlag and Wickings (1975) reported that ratios between 8:1 up to 30:1 steroid to protein were antigenic when BSA was used as carrier. There was no apparent difference in antigenicity between these limits, nor was there good evidence of the ratio within these limits affecting the quality of the antiserum produced. No significant difference in Ab production was found using BSA and haemocyanin suggesting that even when relatively high numbers of steroid molecules are bound to the carrier, provided the number of molecules is greater than eight, there is little advantage in using higher mwt carriers in terms of antigenicity of the steroid-protein complex. However because of the empirical nature of immunisation this may reflect variation in the animal's response rather than the true immunological response to different carriers bearing different numbers of steroid molecules. An _in vitro_ immunisation system (see later this chapter) could provide a better approach to investigating the relative merits of different carrier proteins and molar ratios as the system removes many of the variables inherent in _in vivo_ immunisations.

With ecdysteroid conjugates the carrier proteins most frequently cited were serum albumins and thryoglobulin (see Table 3.1). Horn _et al_ (1976) achieved a ratio of 140:1 using thyroglobulin as a carrier while typical ratios with albumins were around 20:1 (BSA has 60 theoretical sites for conjugation but because of the polypeptide chains 2^{o} structure the folding limits the number to between 20-30 (Kellie, 1975)).

Method of 20-HE-BSA Conjugation

For both polyclonal and monoclonal Ab production in this study a 20-HE conjugate was prepared using the 6-keto group on the steroid B ring as the site of derivatisation. Subsequent protein coupling was by the mixed anhydride technique (Erlanger et al, 1957) using BSA as carrier. BSA was chosen because it has been extensively reported to be an effective carrier protein (Nieshlag and Wickings, 1975).

The method of derivatisation used was that described by Porcheron et al (1976).

Derivatisation: 20-HE-Carboxymethyloxime (20-HE-CMO) Formation

25 mg 20-HE (Simes, Italy) was dissolved in 1.5 ml of pyridine. To this 125 mg carboxymethoxylamine hemihydrochloride (Aldrich) was added. The reaction mixture was incubated at 40°C overnight in a shaking water bath (Borst and O'Connor, 1972) ^{3}H-20-HE (200,000 dpm) was included as a tracer to estimate the recovery (New England Nuclear, 3.8 Ci/mmol). Pyridine was eliminated by repeated washing with 10 ml volumes of benzene followed by evaporation to dryness. Removal of pyridine was confirmed by TLC against a pyridine marker (Merck 60F 254 TLC plates). The preconditioned plates (110°C for 1 hour) were run in 100% chloroform for 90 mins. Spots on the plates were visualised under u.v. light (Mineralite UVSL-58). To the pyridine-free residue 55 ml of ethyl acetate: 30% methanol solvent system was added (10:1 v/v). The 20-HE-CMO was extracted in the organic phase and evaporated to dryness. The residue was taken up in a small volume of ethyl acetate and run on a TLC plate between two 20-HE markers. The solvent system used was methanol, chloroform and water (60:30:5 v/v/v). The preconditioned plate was run for 90 mins and visualised as described above. The major area showing u.v.

absorbance was scraped and eluted in 70% ethanol (v/v). The final recovery was estimated by the radioactivity of the eluted sample and was found to be approximately 35%. A small sample of the product was retained for the u.v. analysis of the final conjugate to estimate the molar ratio of steroid to protein. The 20-HE-CMO had an absorbance maximum at 252 nm.

20-HE Derivative Coupling to BSA

The 20-HE-CMO was dried under nitrogen and dissolved in 100 ul of fresh dioxane and put on ice. 126 umol tri-n-butylamine and 9.5 umol isobutylchloroformate was added to the derivative and the mixture incubated at 0°C for 30 mins. 6mg BSA (Sigma, RIA grade) was added to 216 ul distilled water and allowed to dissolve without stirring. To this solution 144 ul dioxane was added. The water/dioxane/BSA mixture was then added to the reaction vessel at 0°C and incubated at 4°C for 4 hours. To this was added 9.5 ml distilled water and the final solution was dialysed against running water (Erlanger et al, 1959). Following dialysis the solution was lyophilised and stored at 4°C under desiccation. The final yield of conjugate was 19.2 mg. For a schematic representation of the steroid-protein conjugate formation see Fig 3.1.

Characterisation of the 20-HE-BSA Conjugate by u.v. Analysis

The method and conditions of analysis were those described by Erlanger et al, (1959). The molar ratio of steroid to protein was found to be 16:1. According to Nieshlag and Wickings, (1976) this ratio should be sufficiently high to confer antigenicity on the conjugate, in terms of specific Ab production directed against the hapten.

Immunisation Protocols

The antigenicity of the conjugate was tested by attempting to raise both polyclonal and monoclonal Abs.

Polyclonal Ab Production

The protocol described by Vaitukaitis et al, (1971) was used for rabbit immunisation. Briefly, the method involves low doses of immunogen administered at multiple sites subcutaneously. This protocol was chosen because it was less costly in terms of immunogen. Standard protocols would consume mg quantities of Ag and with only 19 mg available for both polyclonal and MAb production this was considered to be unsuitable. 1mg of the 20-HE-BSA conjugate (prepared above) was sonicated with 2.75 ml PBS, 2.75 ml Freunds complete adjuvent (Sigma) and 2.75 ml 1% Tween 60 (Sigma) in PBS. Two New Zealand white rabbits were injected subcutaneously at multiple sites on the back. Thirty injections of 200 ul were administered to each rabbit. Each rabbit received a total of 500 ug of conjugate.

After ten weeks the rabbits were boosted with 100 ug of the conjugate per rabbit in Freunds incomplete adjuvant (Sigma). Six injections were given to each rabbit, four dorsal subcutaneous and one intramuscular into each hind leg.

Assay of Serum Titer

1 ml of blood was removed from the central ear artery of each rabbit and left overnight at 4°C until the serum was expressed. A standard RIA binding assay was performed to find the antiserum dilution that would bind 50% of the radiolabelled ligand (^{3}H-ecdysone).

RIA Binding Curve

Antiserum was diluted with borate buffer (BB) (pH 8.4) containing 1% BSA (Sigma, RIA grade) from 1:50 to 1:12,800 by serial dilution. To 100 ul of each dilution 100 ul BB + 1% BSA containing approximately 4000 cpm ^{3}H-ecdysone (New England Nuclear, 53.6 Ci/mMol) was added. The Ab and radiolabelled ecdysone were incubated at room temperature for 4 hours or overnight at 4^{o}C. The precipitation of bound counts was performed using ammonium sulphate. 200 ul saturated ammonium sulphate (SAS) (BB) at 4^{o}C was added to each incubation tube and vortexed immediately. The tubes were then incubated for 30 mins at 4^{o}C and spun for 15 mins at 4500 rpm at 4^{o}C (Beckman J6). Supernatants were carefully discarded and the pellet washed in 50% SAS (v/v with BB) at 4^{o}C and vortexed then incubated for 20 mins at 4^{o}C. The centrifugation step was repeated. The supernatants were again discarded and the pellet resuspended in 200 ul distilled water and counted in 5 ml of liquid scintillant (Cocktail T, BDH). Radioactivity was quantified in an LKB liquid scintillation counter. Since counting efficiency was consistent at 30%, no quench correction was performed and the results are expressed as cpm. A plot of antiserum dilution against cpm was used to find the Ab dilution required to bind 50% of the cpm.

Results of Polyclonal Ab Production

After two boosts the Ab titer in both rabbits had only reached approximately 1:200 which was considered too low a titer to be useful in terms of a good supply of Ab.

The more Ag boosts that are given the lower the affinity of the resulting antiserum because more B-cell clones are recruited into the response that have a lower affinity than those selected by low Ag levels. Despite this it was considered advisable to continue to boost the animals. However no 2^{o} response was elicited in the two rabbits over the course of the following six months. At this point the decision was made to stop subsequent immunisation as it seemed unlikely that the rabbits were going to respond. Whether this reflected an inadequate immunisation protocol was not immediately clear. It could be that multiple site immunisations using low Ag doses is a less reliable approach to Ab production than standard immunisation protocols. In the first instance low dose immunisation was attractive because the availability of the steroid-protein conjugate was limited and part of it was required for MAb production and the development of a suitable screening assay.

Another factor that could have caused the poor responses seen in the rabbits is the antigenicity of the conjugate used as the immunogen. To investigate the likely reason for the immunogen's failure to elicit good polyclonal Ab production MAbs were raised against the same conjugate using more standard immunisation protocols.

MAb Production Against the 20-HE-BSA Conjugate

Successful hybridoma production depends upon the number of specific B-lymphocytes that are activated in response to Ag and localised in the spleen. The most successful immunisation protocols used with soluble Ags (which are often less potent at eliciting an immune response than insoluble Ags) have involved several

consecutive boosts with Ag in the final four days prior to the fusion (Stahli et al, 1980).

To investigate the type of immunisation protocol required for the 20-HE-BSA conjugate two approaches were taken, one using the type of rigorous protocol described above and the other approach to use a standard scheme consisting of a single, 1^{o}, immunisation and two subsequent boosts.

Immunisation

All 1^{o} immunisations were performed on six week old female BALB/c mice. The Ag used was the 20-HE-BSA conjugate described above.

1 mg of the conjugate was dissolved in 1 ml PBS and sonicated briefly. 500 ul of the suspension was taken up in a 1 ml syringe and connected by fine plastic tubing to another 1 ml syringe via 26G needles containing 500 ul of Freunds complete adjuvent (Sigma). Mixing was achieved by repeated transfer between the two syringes and was considered adequate when the emulsion formed discreet droplets when placed on water. Two mice were injected, IP with 0.2 ml of the emulsion which was equivalent to 100 ug of the steroid-protein conjugate.

After three weeks one mouse was given a boost of 100 ug of the conjugate (IP) in 0.2 ml PBS, and this was repeated ten days later and the fusion performed four days after the final boost. A second mouse was immunised in the same way and boosted after three weeks but given a rigorous immunisation protocol (Stahli et al, 1980), that is summarised in Table 3.2, up until the day of the fusion.

Immediately prior to each fusion a small volume of blood was removed by tail bleeding from each mouse and the serum assayed in the screen assay (see later this chapter). The serum from the mouse given a standard immunisation protocol was not found to be 'positive' under the assay conditions used. The serum from the mouse that had received the rigorous immunisation regime was found to have anti-conjugate Abs under the ELISA conditions used for the other mouse and gave absorbance values ten times greater than those with normal (that is, unimmunised) mouse serum.

Fusion of Immunised Mouse Spleen Cells with P3/X63-Ag8.653 Myeloma Cells

The methods used are those described in detail in chapter II. This applies to both fusions and subsequent selection and cloning techniques.

Screening Assay for MAb Against 20-HE-BSA Conjugate

A good screen for MAb is one that enables multiple culture supernatants to be assayed simultaneously in a rapid and reliable fashion. RIA was considered for the screening of anti-conjugate MAbs but was rejected on the basis that it is relatively time-consuming and involves the manipulation of multiple tubes, separation of bound from unbound steroid by centrifugation and liquid scintillation counting. Enzyme-linked immunosorbent assays (ELISA) are rapid and can be conveniently performed in multi-well plastic plates enabling several tens or hundreds of culture supernatants to be assayed simultaneously. Such an assay was developed in the first instance using the polyclonal anti-ecdysone

antiserum, DUL-2 (Gift from Professor J. Koolman, Philipps-Universitat, Marburg, FRG) to establish assay conditions and then adapting the assay for use with mouse-derived Ab in culture supernatants.

Principles of ELISA

The Ag is rendered insoluble by attaching it to the plastic of the assay wells or assay tubes. Because the capacity of different Ags to associate with plastic is very variable the coating conditions must be established for each individual Ag. In some assays the Ab rather than the Ag can be used to bind to the plastic, but because the screen in this project used the Ag as coating substance only this particular type of assay protocol will be discussed.

Once attached to the well the Ag is in a state of dynamic equilibrium as the Ag-plastic association does not involve covalent bond formation. The supernatants to be assayed are then placed in the Ag coated assay wells and incubated for a predetermined length of time. A second Ab is then added to the wells which is specific to mouse Ig. This second Ab is coupled to an enzyme such as alkaline phosphatase or horseradish peroxidase. The bound enzyme conjugate is visualised by incubation with an appropriate enzyme substrate that gives a coloured product. High absorbance values relative to appropriate controls indicates that Ab is present in the culture supernatant that is directed against the coating Ag. For a schematic representation of Ab binding in an ELISA see Fig 3.2.

ELISA Methodology

ELISA plates were coated with the 20-HE-BSA conjugate at a

predetermined concentration. Culture supernatants were screened against these wells and also BSA coated wells to discriminate between MAbs directed against the carrier rather than the hapten.

96 well flexible activated pvc ELISA plates (Flow-Titertek) were coated with 100 ul of a 2 ug per ml* solution of 20-HE-BSA conjugate in PBS 24 hours prior to the assay. In some later experiments and screens free ecdysteroid was found to be suitable as a coating solution if used with the flexible pvc plates. The optimal coating solution was found to contain 4 ug per ml* ecdysteroid in PBS. These two coating systems were often used interchangably. The coating solutions were incubated in the plates at 4°C overnight covered with 'clingfilm'. On the day of the screening assay the coating solution was tipped from the wells and 100 ul of PBS + 1% casein added to block any binding sites still available in the wells. The blocking solution was incubated at 37°C for 30 mins. The solution was discarded and the plates washed with 3 x 5 min changes PBS + 0.05% Tween 20 (Sigma). 50 ul of the hybridoma culture supernatants to be tested were placed in each well and diluted with 50 ul PBS + 1% BSA (this reduces the number of anti-BSA MAbs detected). The supernatants were incubated for two hours at 37°C or overnight at 4°C. Again the supernatants were discarded and the wells washed as described above. The enzyme-Ab conjugate used was rabbit anti-mouse IgG (whole molecule) - alkaline phosphatase (Sigma) and was diluted with PBS + 0.05% Tween 20 to give a final concentration of 1:1000 (v/v). 100 ul of this solution were placed in each ELISA well and incubated for one hour at 37°C. The conjugate was then discarded and the wells washed as described above. The enzyme substrate 'Sigma 104' (Sigma) was made up immediately prior to use at a concentration of 1 mg per ml in 10% (w/v) diethanolamine buffer (pH

* See appendix number 2 for optimisation of coating concentrations.

9.6). 100 ul of the substrate solution was added to each well and the plate incubated at room temperature for one hour and the absorbance value for each well ascertained using an ELISA photometer plate reader (Flow-Titertek) using a 405 nm filter. Positive wells were designated to be those with absorbance values at least ten times greater than background which were wells containing an 'irrelevant' mouse MAb supernatant as first Ab in the assay.

Results of MAb Production Against the 20-HE-BSA Conjugate

Two fusions were performed. One mouse had received one 1^{o} immunisation and two boosts, the final boost being administered four days prior to the fusion. The second mouse had been given a rigorous immunisation regime detailed in Table 3.2. The fusions were plated out into 500 wells.

The first fusion had a fusion frequency (percentage of wells showing hybridoma growth) of 75% and of these wells only three were positive (0.8%) and producing Abs directed against the conjugate. Of these three wells only one contained hybridomas secreting Ab directed against the steroid, the other two exhibited anti-BSA activity.

From the second fusion a fusion frequency of 80% was achieved. Twentyfour wells (6%) were positive in the ELISA screen and of these six showed anti-ecdysteroid activity rather than anti-BSA activity (1.5%).

From the results from two fusions, bearing in mind the small sample size, it would seem that the rigorous immunisation protocol was more effective in eliciting an immune response against the immunogen relative to the standard immunisation protocol also used.

Evaluation of the Anti-Ecdysteroid-Secreting Cell Lines

The seven cell lines were partially evaluated using culture supernatants as a source of Ab. Ig class determination used the mouse MAb typing kit (Serotec) described in chapter II. All seven cell lines were found to be secreting Ab of the IgM Ig class. The stability of MAb under storage conditions was investigated (see chapter II). In the case of three of the seven cell lines, Ab was found to be unstable if subjected to repeated freezing and thawing. These cell lines MAbs were therefore stored 1:1 (v/v) with glycerol at -20°C. Under these conditions they remained stable for several months. The remaining four cell lines Abs were stable under storage at -20°C.

The capacity of the MAbs to bind ^{3}H-ecdysone and ^{3}H-20-HE was investigated using the RIA binding assay described elsewhere in this chapter. Under the assay conditions used all seven cell lines Abs failed to precipitate radiolabelled steroid to an extent that significantly differed from background. To test if this was simply a function of Ig concentration the culture supernatants were concentrated 100 times by ammonium sulphate precipitation (see chapter II). In all cases the MAb still failed to precipitate ^{3}H-ecdysteroid.

The affinity of the MAbs was investigated qualitatively by investigating the intra assay variation using the ELISA screen. Multiple replicates (n = 10) of each culture supernatant were assayed in parallel.

Voller et al (1979) reported that replicates in ELISA should fall within ± 10% of the mean and that variation beyond these limits can be due to differential Ag coating, differential washing or Ab affinity. The MAbs were therefore compared within the same assay to

DUL-2 polyclonal antiserum which served as a characterised, high affinity anti-ecdysteroid polyclonal Ab in the absence of a suitable MAb to act as control. DUL-2 was included to ensure that any variation observed was due to Ab affinity not to variability inherent in the assay protocol. The data is summarised in Table 3.3.

The variability of the MAb in intra assay replicates was large (see Table 3.3). The variation indicates the relative strength of Ag:Ab interaction in this system. Whereas DUL-2 gave intra-assay variability of less than $\pm$ 10% the seven MAbs gave much higher levels of variation. All the anti-ecdysteroid MAbs had too low an affinity to give variation within an acceptable range of $\pm$ 10% of the mean and as a result were unsuitable for use in ELISA and RIA under the conditions described. This low affinity could perhaps explain why the MAbs failed to precipitate ^{3}H - labelled ecdysteroids under RIA conditions since there is no structural reason why IgM class Ab should be unable to precipitate labelled steroid.

It was concluded that the seven cell lines generated from the two mouse spleen cell fusions were of too low an affinity to be useful for determining ecdysteroid titers using ELISA and RIA from the relative numbers of positive cell lines generated by the two immunisation protocols. It was concluded that the conjugate was not highly antigenic and required a rigorous immunisation regime to elicit a reasonable response to the immunogen. The doses of Ag administered in this protocol were very high for mouse immunisation and therefore costly in terms of the quantity of conjugate used. It was therefore decided that a second attempt would be made to generate MAb to the steroid protein conjugate of a sufficiently high

affinity using in vitro immunisation. This has the advantage of using a low single dose of immunogen. As the immunisation is performed under standardised culture conditions many of the variables inherent in in vivo immunisation are eliminated.

In vitro Immunisation

Introduction

The first study concerned with immunisation in vitro was undertaken as early as 1912 (Carrel and Ingebrighsten, 1912). Anti-goat red blood cell Abs were successfully generated from B-cells derived bone marrow and lymph node fragments maintained in culture. This type of approach lends itself to the study of cellular interactions that occur when an immune response is initiated. Primary immunisation using cultured, dissociated murine spleen cells was first performed in the 1960's (for review see Fishman, 1969) and along with studies using human cells (Hoffman et al, 1973) the involvement of several distinct cell populations was demonstrated in the humoral immune response. The first application of in vitro immunisation for the generation of hybridoma cells was in 1978 (Hengartner et al, 1978) and many incidences of successful MAb have since been reported using this technique (reviewed by Reading, 1982).

The advantages of in vitro as opposed to in vivo immunisation can be briefly summarised as follows. The entire immunisation process only takes 5 days, a defined Ag concentration can be maintained in the culture medium and it is also possible to raise Ab to 'self' Ags which in vivo would be subjected to tolerance/ suppression mechanisms. However, because the response in vitro is essentially 1^{o} in nature there is a dominance of IgM Abs relative

to IgG which may theoretically lead to a predominance of lower affinity Abs than in in vivo immunisation. For an extensive discussion of the formulation of the culture conditions and applications to which the technique has been applied see review by Reading (1982). The technique has been of particular value in eliciting specific Ab production against Ags which are either weakly antigenic or would be required in mg quantities for in vivo immunisation. One example is anti-calmodulin Ab production (Pardue et al, 1983). For this particular reason it was of interest to see if this technique could provide a more effective means of raising specific MAb directed against the 20-HE-BSA conjugate, with more conservative quantities of Ag being required.

In vitro Immunisation Methodology

The methods used in this study were essentially those described by Reading (1982) with the exception that mixed thymocyte conditioned medium was replaced with peritoneal exudate cells (Murakami et al, 1982).

Culture Medium and Conditions

Supplemented Dulbecco's Modified Eagle's Medium (SDMEM) was used throughout and was made up as follows: DMEM (1 litre) containing high glucose (4.5g/litre) (GIBCO) was supplemented with 1% MEM non-essential amino acids (w/v) (GIBCO), 1 mmol sodium pyruvate (GIBCO), 50 umol 2-ME (GIBCO), 30ummol hypoxanthine (SIGMA), 3 umol thymidine (SIGMA) 26 mMol sodium bicarbonate (GIBCO), 18 mmol HEPES (GIBCO) 20% (v/v) FCS (Flow), and 6 mmol L-glutamine (GIBCO).

2-ME was found by Click et al (1972) to enhance in vitro Ab production and Opitz et al (1977) found 2-ME treated FCS was

mitogenic for T-lymphocytes and could therefore functionally substitute for macrophages. Goodman and Weigle, (1977) reported that 2-ME and FCS resulted in polyclonal B-cell activation; for these reasons it was included in the culture medium.

The medium was prepared as follows: The contents of the 1 litre package of DMEM was dissolved in 1.1 litres of double distilled water and the HEPES and sodium bicarbonate were added while stirring gently. 10ml of each of the following stock solutions were then added: 100 mM sodium pyruvate, 5 mM 2-ME, 100 x non-essential amino acids, 0.3 mM thymidine and 3 mM hypoxanthine. The pH was adjusted to 7.3 using 1N NaOH/1N HCL. The osmolarity was adjusted to 290 mosmoles/litre. Finally, the medium was sterilised by filtration (0.2um- Millipore) under slight vacuum. Once sterile FCS was added (20% v/v) and the 6 m L-glutamine along with tylocine ($.5 \times 10^{-5}$ M.).

In vitro Immunisation

The Ag used was the 20-HE-BSA conjugate prepared as described earlier in this chapter. 5 ug of the conjugate was mixed with 100 ug BSA and dissolved in 5 ml SDMEM and sterilised by filtration (Millpore: 0.2u) (BSA was included to reduce the likelihood of the steroid protein conjugate adhering to the filter).

The spleen of an eight week old unimmunised female BALB/c mouse was removed asceptically and perfused with PBS (as described in chapter II). The spleen cells were spun at 200g for 10 mins, (MSE centrifuge), the PBS discarded and the cells resuspended in 10 ml SDMEM and placed in a 25 ml culture flask (Nunc). Macrophages were prepared (see chapter II) and were added to the spleen cells to give a final cell density of approximately 3 x 10^5 cells per ml.

Finally the Ag in SDMEM was added to the culture flask to give a total volume of 15 ml. The flask was shaken gently and incubated for 5 days (for incubator conditions see chapter II). After about 3 days the cells could be seen to be clumping with large blast cells clustering and attached to adherent cells. By day 5 this was widespread amongst the cultured cells and at this stage the cells were considered ready for fusion (Reading, 1982).

Fusion

The method of fusing the spleen cells with the myeloma cells was identical to that described in chapter II. Following fusion the cells were plated out into 4 x 96 well plates previously prepared with peritoneal macrophages and were incubated for 14 days. The cells were plated out in SDMEM plus 4 x 10^{-2}M aminopterin on the day of fusion and after two weeks the aminopterin was omitted from the SDMEM for one week and then the cells were gradually transferred to RPMI medium for long term culture.

Results of in vitro Immunisation

In this study the cells were screened after 21 days for anti-ecdysteroid activity using the ELISA described previously in this chapter. Plates were blocked with 1% casein in PBS. The fusion frequency obtained was significantly lower than in standard *in vivo* immunisation fusions, that is 25% as opposed to approximately 80% or greater. This was probably due to the spleen cell viability decreasing during the period of culture with the immunogen prior to the fusion. The results of the *in vitro* immunisation are summarised in Table 3.4.

Of the approximately 400 wells which received post-fusion cells

100 showed hybridoma growth, that is a fusion frequency of 25%. Of those wells that were assayed 9% were 'positive' in the ELISA screen and were directed against ecdysteroid rather than BSA. Some of the characteristics of the cell lines MAbs are summarised in Table 3.4. All nine cell lines secreted IgM class Ig. This would be anticipated to result from in vitro immunisation (Pardue et al, (1983) found 80% of Ig secreting hybridomas generated by in vitro immunisation secreted antibody of the IgM class of Ig). None of the cell lines MAb precipitated radiolabelled ecdysteroid under RIA conditions with bound cpm not significantly differing from background in all cases. Even when the culture supernatants were concentrated by ammonium sulphate precipitation to approximately 100 concentration no significant binding of radiolabelled ecdysteroid occurred. In intra assay replicates the SE was large. In all cases the variation observed was greater than 25% as opposed to 7.5% with the DUL-2 polyclonal Ab. These results indicate that in vitro immunisation is a very good method of generating anti-ecdysteroid MAb to low levels of Ag. Mg quantities of conjugate were required in vivo to elicit 1.5% of fusion wells showing hybridoma growth that secreted MAb directed against the hapten. 9% were yielded by in vitro immunisation using only 5 ug of conjugated ecdysteroid. The relative affinities of the MAbs appeared too low to give an intra assay variation below 10%. In all cases the variability of replicates was greater than 25%. Again it appears that the affinity of the isolated MAbs is too low to be useful in ELISA and RIA for the assay of ecdysteroids.

IgG Abs can be of higher affinity than IgM Abs. To ensure higher affinity Mabs against the ecdysteroid conjugate two possibilities were considered. Multiple fusions could be performed

using in vitro immunisation to generate hundreds of MAb of which it would be predicated some should be of high enough affinity to be useful in assay systems. Alternatively the in vitro immunisation protocol could be modified in such a way that the ratio of IgM:IgG class Abs would be closer to 1:1. Pardue et al, (1983) rechallenged spleen cells during in vitro culture prior to the fusion and generated IgM:IgG Abs with equal frequency. It was decided that this approach could be useful in the generation of higher affinity anti-ecdysteroid MAbs.

Repeated Challenge With Ag in the in vitro Immunisation System

The protocol already described for single Ag exposure in vitro immunisation was repeated. After 4 days initial culture with 5 ug of the 20-HE-BSA conjugate the spleen cells were spun down and cultured for 3 days in SDMEM in the absence of Ag. On day 8 5ug of the 20-HE-BSA conjugate was introduced into the culture medium and cultured for a subsequent 3 days. Again the cells were observed to form clumps and the fusion was performed on day 12.

The fusion frequency was only 20% and presumably reflects the low viability of spleen cells during the extended period of cell culture. Of the eighty wells showing hybridoma growth only one cell line, 2A10 secreted MAb directed against the hapten rather than the carrier as determined by ELISA (see Table 3.5).

Discussion

The aim of the work described in this chapter was to generate anti-ecdysteroid MAbs. An ecdysteroid-protein conjugate was successfully synthesised and its antigenicity tested in polyclonal and MAb production. It proved to be only weakly antigenic and did

not elicit vigorous Ab production in rabbits or mice under the immunisation schemes described. In mice a very rigorous immunisation protocol was found to be necessary to induce specific Ab production but required a very high total dose of immunogen. An in vitro immunisation system was therefore attempted to combat the requirement for large quantities of Ag and proved to be highly successful even using 1/300 the dose of immunogen required to produce an inferior response (as determined by the percentage of anti-hapten cell lines generated) by in vivo immunisation.

The MAbs secreted by the hybridoma cell lines that showed anti-ecdysteroid activity all proved to be of relatively low affinity. They were therefore unsuitable for the determination of ecdysteroid titer in ELISA or RIA because of the high levels of intra-assay variation they showed and their inability to precipate ^{3}H labelled ecdysteroid under RIA conditions. However critical criteria which are essential for Ab production against soluble steroid-protein conjugates were identified. Each will now be discussed in more detail.

The Antigen

The 20-HE-BSA conjugate synthesised had an apparent steroid/protein molar ratio of 16:1 as determined by u.v. absorption, which should theoretically be satisfactory as an immunogen (Nieshlag and Wickings, 1975). However it proved to be a poor antigen in polyclonal Ab production and only antigenic if used in very rigorous immunisation regimes in MAb production. It is possible that the u.v. absorption analysis method used to estimate the coupling ratio overestimated the amount of steroid attached to the BSA. Any free steroid that had failed to be physically separated

from the protein bound steroid during the conjugate production could cause an over-estimation to be made of the number of steroid molecules that were covalently bound to the steroid. This is supported in the literature by the successful anti-ecdysteroid Ab production resulting from conjugates with hapten-carrier ratios of below 8:1 (Borst and O'Connor, 1974) which suggests the ratios in the literature may not be a true representation of the molar ratio. The problem of separating conjugated steroid and free steroid could have been responsible for the relatively low antigenicity of the conjugate used during this study. This explanation of weakly or non-immunogenic ecdysteroid protein conjugates has been expressed by other workers. (Dr. H. H. Rees, Dept. Biochem. Univ. Liverpool. personal communication). See appendix number 3 for further details.

Immunisation

Immunisation protocols are notoriously empirical and are subject to the interaction of many variables such as strain of animal, route of administration of the immunogen as well as the immunogen itself and how rapidly it is degraded by the host animal. For polyclonal Ab production using low Ag doses administered at multiple sub cutaneous sites perhaps more rabbits should have been immunised to guarantee a response. Alternatively it could be a reflection of the quality of the immunogen (see above). With MAb production against the soluble Ag only rigorous immunisation protocols proved to be successful in inducing specific Ab production. A higher level of specific B-cell stimulation was observed using 1/300 the dose of Ag using an in vitro immunisation. This has implications for the production of MAb to both highly impure Ag and also to pure Ag available in very low quantities. The technique is quick and more

effective than intensive *in vivo* immunisation. It is possible that repeated challenge with Ag can be a way of inducing a more equal ratio of IgM to IgG Abs. However it is undoubtedly a question of balancing the length of time the cells are maintained in culture with the decreasing cell viability with time. If these two parameters could be optimised this would offer a successful approach to immunisation as it would result in Abs comparable to those produced by *in vitro* immunisation in terms of specificity and Ig class but would allow the majority of the variables inherent in *in vivo* systems to be eliminated.

Abs Produced

The MAbs produced were all of the IgM Ig class, all showed specificity towards the hapten (20-HE) rather than the carrier protein (BSA). However they were all characteristically of low affinity. In multiple intra-assay replicates the variability observed in the ELISA was always greater than 25%. Typical intra-assay variation using high affinity Ab was in the order of 10%, as was observed using the DUL-2 polyclonal Ab directed against ecdysteroid. The result of this relatively low affinity was that no isolated cell lines were identified synthesising Ab suitable for use in ELISA. When tested for their capacity to precipitate tritiated ecdysteroid under RIA conditions no Abs were found that gave binding significantly different from background. Whether this is attributable to low affinity or to their identity as IgM Ig class Abs is not apparent. IgM Ab is the first Ig class synthesised in response to a 1^{o} immunisation and they may show lower affinities relative to IgG Ab (see chapter II). It is probably due to chance that all the isolated cell lines Ab were of insufficiently high

affinity to be useful in ELISA and RIA. In the case of *in vivo* immunisation a greater proportion of IgG Abs might be generated by extending the period of time between the 1^{o} and 2^{o} immunisations. However because of the high doses of Ag required to induce a response *in vivo* this would have led to the selection of low affinity clones. With *in vitro* immunisation it is probably true that high affinity Ab can only be guaranteed to be generated if multiple fusions are performed to produce more hybridomas and that the effects on affinity caused by different Ag doses are fully investigated.

Conclusions

A number of methods of polyclonal and monoclonal Ab production were used to prepare anti-ecdysteroid Ab. All used a 20-HE-BSA conjugate preparation that had an apparent coupling ratio of 16:1. While the results enabled a number of conclusions to be drawn about the relative importance of variables affecting the success of Ab production, no satisfactory Abs (suitable for use in immunoassays) were identified. It is likely that the main problem experienced was the poor antigenicity of the 20-HE-BSA conjugate. It may be that the ratio of steroid to protein in the conjugate was overestimated. There are many problems inherent in the method of analysis of protein steroid conjugates. If the bound and free ecdysteroid are not fully separated then analysis will give a falsely high ratio of steroid molecules to protein molecules bound.

The immunisation protocol used with the weakly antigenic soluble antigen was found to be critical. In polyclonal Ab production multiple site, low dose approaches (Vaitukaitis *et al*, 1971) were not found to be very successful and perhaps this method is only

satisfactory for good quality immunogens. For MAb production a 1^{o} immunisation followed by two boosts failed to elicit detectable humoral Ab in the ELISA. Only when intensive boosting using high doses of immunogen was used was circulating Ab detectable. MAb was isolated but found to be of low affinity. *In vitro* immunisation proved to be highly successful even with 1/300 dose used for *in vivo* immunisation. This has exciting implications for anti-ecdysteroid MAb production and is the most satisfactory method of immunisation to be followed up in the future. The MAbs generated were of low affinity but in numbers greater than those generated by high dose *in vivo* immunisation.

The problem of low affinity was disappointing but is probably not insurmountable. To attempt to elicit more IgG Abs an *in vitro* approach was taken that incorporated a second Ag challenge. Only a single cell line was generated that was secreting MAb specific to the hapten. This no doubt reflects the decreasing spleen cell viability during prolonged culture. However if the protocol could be optimised in terms of temporal viability of spleen cells under culture conditions and the time course necessary for *in vitro* 2^{o} response induction this could prove to be a very valuable method of inducing specific MAb. It would also have the potential to generate high affinity Ab which would prove useful for the determination of ecdysteroid levels in biological samples in immunoassay systems.

Finally, the screen developed (ELISA) for the MAbs could provide a very useful alternative to RIA if used in conjunction with suitable Ab as it has many advantages over radiometric analysis (see earlier this chapter).

Table 3.1 Characteristics of some Ecdysteroid-Protein Conjugates and their Antibodies.

References:

1. Borst and O'Connor (1972)
2. Lauer et al (1974)
3. De Reggi et al (1975)
4. Porcheron et al (1976)
5. Horn et al (1976)
   Reum and Koolman (1979)
6. Maroy et al (1977)
7. Spindler et al (1978)

HAPTEN	CARRIER	MOLAR RATIO Steroid: protein	RELATIVE AFFINITY Ecdysone : 20-HE	REFERENCE
6-Carboxymethyloxime of 20-HE	BSA	3.3:1	1:1	1
2,3,22-monohemisuccinates of 20-HE	HSA	20:1	20:1	2
2-monohemisuccinate of 20-HE	HSA	11:1	1:1	3
6-carboxymethyloxime of 20-HE	BSA	20:1	N.A.	4
22-hemisuccinate of 20-HE	Thyro globulin	140:1	10:1	5
Polypodine B 6-carboxymethyloxime	RSA	8:1	1:1	6
Inokosterone-26-oic acid	Thryo globulin	164:1	1:0.3	7

BSA Bovine serum albumin
HSA Human serum albumin
RSA Rabbit serum albumin

Table 3.2 Rigorous Immunisation Protocol for Soluble Ag

Days prior to Fusion	Ag dose (ug)	Route	Injection System
4	200	IP	PBS
3	400	IP	PBS
2	400	IP	PBS
1	400	IP	PBS
0	Fusion	-	-

Table 3.3 Some Characteristics of the anti-ecdysteroid-secreting cell lines.

Cell line	Ig Class	RIA ^{3}H-ecdysteroid precipitation (% bound)	ELISA Intra assay variation (n = 10) Mean absorbance values ± SE $\bar{x}$	± SE
2A2	IgM	0	0.62	0.33
5E8	IgM	0	0.39	0.15
4C12	IgM	0	0.64	0.42
4C8	IgM	0	0.28	0.09
5C4	IgM	0	0.35	0.21
3E9	IgM	0	0.47	0.18
4B2	IgM	0	0.59	0.17
DUL-2 (1:500)	Poly clonal	50	1.30	0.10

Table 3.4 Some Characteristics of the MAbs resulting from *in vitro* immunisation which exhibit anti-ecdysteroid activity.

Table 3.5 MAb Generated by Repeated Challenge with Ag *in vitro*.

Cell line	Ig Class	^{3}H-ecdysteroid precipitation (% bound)	Intra assay variation (n = 10) Mean absorbance values ± SE x	± SE
1A12	IgM	0	0.35	0.16
1B1	IgM	0	0.47	0.25
1C5	IgM	0	0.61	0.25
1H1	IgM	0	0.25	0.07
2B1	IgM	0	0.30	0.16
2H11	IgM	0	0.19	0.12
3A9	IgM	0	0.44	0.19
3A12	IgM	0	0.32	0.11
3B6	IgM	0	0.38	0.13
DUL-2 (1:500)	Polyclonal	50	1.20	0.09

0% counts bound = not significantly different from background.
Background was designated 0% bound.

Cell line	Ig Class	^{3}H-ecdysteroid precipitation (% bound)	Intra assay variation (n = 10) Mean absorbance values ± SE x	± SE
2A10	IgM	0	0.42	0.17

Fig 3.1 Schematic Representation of Steroid Protein Conjugation

CONJUGATION OF 20 – HYDROXYECDYSONE TO BSA (MIXED ANHYDRIDE METHOD)

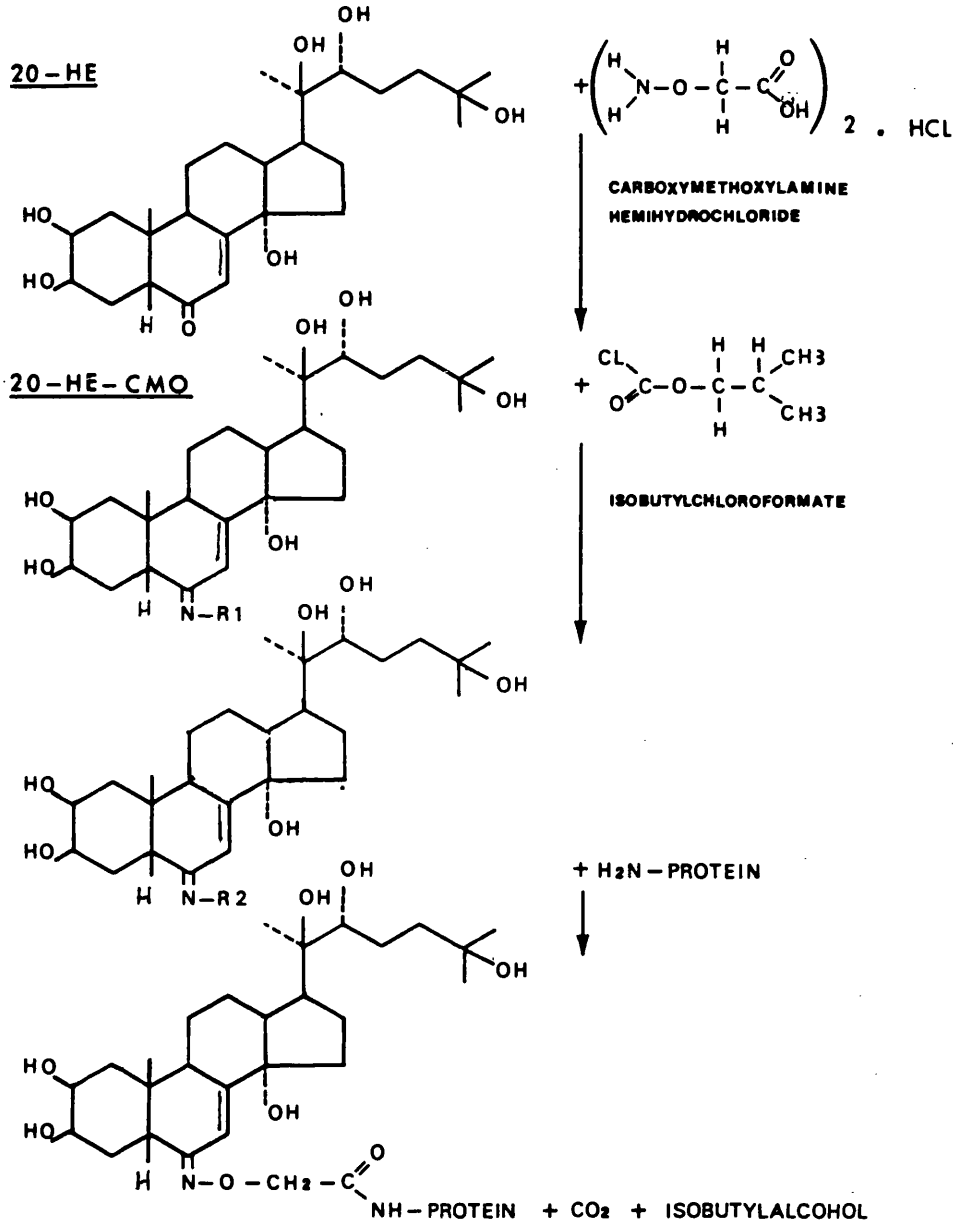
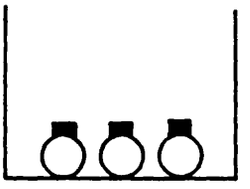
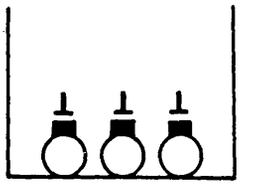


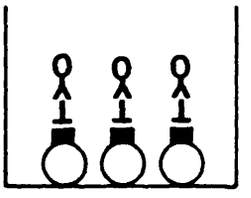
Fig 3.2 Schematic Representation of the ELISA



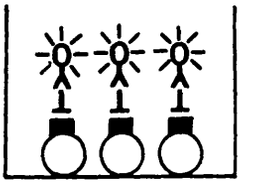
1. COATING



2. INCUBATION WITH PRIMARY ANTISERUM



3. ADDITION OF SECOND ANTIBODY AND INCUBATION



4. ADDITION OF CHROMOGENIC SUBSTRATE

BSA

ECDYSONE

BSA-20-HE

ANTI-ECDYSONE ANTIBODY

ANTI-MOUSE ANTIBODY

ALKALINE PHOSPHATASE

CHROMOGENIC REACTION PRODUCT

Chapter IV: Monoclonal Antibodies Directed Against Prothoracic Gland Antigens

Introduction

MAbs are useful tools for the study of uncharacterised Ags present in crude tissue preparations. As discussed in chapter I, it is of considerable interest to look at the profile of MAbs that can be elicited in response to PG cells and to attempt to define functions for novel Ags which are of potential interest in terms of the gland cells structure and function. For these reasons mice were immunised with intact PGs and the resulting MAbs screened by indirect immunofluorescence techniques against PG sections.

This chapter discusses the range of staining patterns that were observed with the MAbs elicited, and details the investigation of some structural Ags that occur within the cells and in the surrounding extracellular matrix. The possible implications of the occurrence of such Ags and their probable molecular nature is considered. An Ag recognised by MAbs produced from the same fusion which may be an important molecule in the regulation of the PG is discussed in chapter V.

Prothoracic Glands

The functional aspects of the PG of Manduca and other insects have already been given a detailed review in chapter I. However, it is important in the context of this chapter to consider the morphology and ultrastructure of these endocrine glands.

The PG exhibit considerable anatomical variation between different insect groups, in particular with respect to the location and organisation of the glands within the animal (Herman, 1967). In Manduca the PGs are situated at the prothoracic spiracles but extend anteriorally in the form of a chain of cells. The total number of cells in each gland remains constant throughout the fifth instar and

is approximately 330 (Stott, 1983). The morphology of the PG of Manduca can be seen in the scanning electron micrograph (SEM) which depicts the glands in situ in a W + 2 fifth instar caterpillar (see Fig 4.1). (An SEM photograph of some PG cells at a higher magnification can be seen in the frontispiece). For the methods of tissue preparation for EM see Appendix 1. The extent of the connective tissue sheath which encases the cells is clearly visible. This sheath is extracellular in nature (Beaulaton, 1968) but its molecular composition is poorly understood. It serves both to anchor and support the cells within the prothorax but is also a tissue barrier between the PG cells and the surrounding haemolymph. Although the extent to which haemolymph components are restricted in their access to the gland cells by the sheath is probably not great it does prevent direct contact between the gland cells and haemocytes. All nervous and tracheal supplies to the glands have to cross the sheath in order to connect with the gland cells.

Ultrastructure of the Prothoracic Gland Cells of Manduca

The most striking feature of the gland cells is the extensive peripheral channel system (PCS) which is formed by the invagination of the plasma membrane (Fig 4.2). The extent of the PCS varies during the course of development and in Manduca has been correlated with the fluctuations in ecdysteroid titer (Sedlak et al, 1983). The PCS becomes more extensive when the haemolymph ecdysteroid titer is high and it has been suggested that this serves to increase the cells surface area and probably indicates that the glands are actively secreting ecdysone (Sedlak et al, 1983). Within the PCS there are often membrane inclusions which consist of vesicles enveloped within a membrane and are termed multivesicular sacs (mvs). The cytoplasm of the PG cell does not include a great number of organelles although mitochondria are relatively abundant and smooth ER forms a component of the cytoplasm in some species, eg, Bombyx mori (Beaulaton, 1968). The nucleus is highly irregularly shaped (Fig 4.3). The mvs are thought to be important in the transport of ecdysone from the glands into the haemolymph by exocytosis. The localisation of sterols in the PG has been studied in Galleria mellonella (Blazsek and Mala, 1978) and were found to be localised in the micro-vesicles. In the same study it was postulated that sterols enter the cells by endocytosis and then, during ecdysone secretion, the steroid is released by exocytosis from the vesicles. There is evidence that in Manduca the mvs lose inner membrane structures at the time that PTTH acts on the PG (Sedlak et al, 1983). This would be analogous to the intracellular events seen during the release of corticosteroid from the vertebrate adrenal cortex in response to ACTH, a tropic vertebrate peptide. Here, corticosteroids have been postulated to be dissolved in lipid and to leave the cell by exocytosis (Rhodin, 1971) and it is possible that this is a widespread method of steroid release in vertebrate and invertebrate steroid secreting cells.

There are other cellular events in PG cells that correlate with ecdysteroid titer changes, for example the matrix of the mitochondria in Manduca PG cells become more electron dense during the fifth instar as ecdysteroid levels increase (Sedlak et al, 1983). This suggests that perhaps biosynthesis of ecdysteroid during the fifth instar is due, at least in part, to mitochondrial enzymes. The major peak of ecdysteroid in the final larval instar of Manduca (see Fig 1.2, chapter I) coincides with the elaboration of the rough endoplasmic reticulum (RER). Sedlak et al (1983) postulated that proteins required for reformation of the plasma membrane could be synthesised at this time as pupal PG in Manduca lack the PCS (Sedlak et al, 1983). The likelihood that the proteins synthesised on the RER were utilised for ecdysone transport was deemed unlikely in the same study because high ecdysteroid levels were detectable in the haemolymph prior to RER whorl formation. However some caution should be applied to the interpretation of ultrastructural correlation with ecdysteroid titer in the absence of knowledge about the biochemical and functional nature of the RER proteins. Until then the true relationship between structure and function remains open to conjecture as it is not easy to separate causation from correlation in structural studies.

Monoclonal Ab Production

An eight week old female BALB/c mouse was immunised IP with 10 pairs of intact W + 2 PG in 0.25 ml PBS. After four days the spleen was removed from the mouse and the fusion performed using the protocol detailed in chapter II. Culture supernatants were screened immunocytochemically using standard 5 um wax sections of PG (W + 2) which had been fixed in 4% formaldehyde overnight and dehydrated through an alcohol series, cleared in xylene and embedded in paraffin wax. The screening protocol is outlined below. The fusion frequency was 89%.

Screening Protocol

Using a diamond pen a circle of approximately 1 cm diameter was etched around the wax PG sections on microscope slides. The slides were dewaxed in 2 x 5 min changes of xylene and hydrated down an alcohol series into PBS. The tissue sections were left in the buffer

for 30 mins and then blocked in a 2.5% solution of normal rabbit serum (Serotec) in PBS for 30 mins. The excess blocking solution was discarded and the slides dried with filter paper, with the exception of the area bordered by the etched circle. To this area 100 ul of test culture supernatant was applied and the slides incubated for 60 mins at room temperature in a humidified chamber. The slides were washed in 3 x 5 min changes of PBS and 100 ul of a 1:40 dilution of rabbit anti mouse-FITC conjugate (Sigma) in PBS was placed on the test area and incubated for 30 mins at room temperature in a humidified chamber. The washing step was repeated and the slides counterstained for 2 mins in a solution of Evans' blue (Sigma) in PBS (50 ul of a 1% solution of Evans' blue in 50 ml of PBS) and mounted using a drop of mounting fluid which reduces photobleaching during visualisation (see below), covered with a glass coverslip and examined immediately. A Zeiss epi-fluorescent (incident light) microscope was used with a 495nm filter. Photography was performed using a 400 ASA Ektachrome film (Kodak) using a standard exposure time of 10 secs for both experimental and control slides.

Mounting Fluid for Fluorescence Microscopy

100 mg of para-phenylendiamine (Sigma) was dissolved in 10 ml of PBS and to this was added 90 ml glycerol. The pH was adjusted to 8.0 using carbonate/bicarbonate buffer pH 9.0. The resulting fluid was aliquoted and stored in the dark at -20^{0}C. (After Huff et al, 1982).

Results of the Screen for MAb Directed Against the PG of Manduca

From the range of staining patterns observed during the screening

of the MAbs some representative, selective, staining patterns will now be discussed and represented photographically. Control preparations using an 'irrelevant' MAb as first Ab were always run in each assay and photographed for the same exposure time (10 secs) as 'experimental' supernatants. In all cases control photographs showed no visible staining at all and so have not been represented photographically in this thesis. The Abs have been categorised on the basis of the region of the PG cell to which they are specific. Peripherally staining MAbs were the most numerous. Representative MAb staining patterns for the cellular regions are shown. The general structure of the PG gland cell is shown to facilitate interpretation of selective FITC staining by the MAbs. The gland cell sections have been stained for material with a high carbohydrate content to highlight the PCS and sheath. The staining was with PAS (Periodic acid, Schiffs reagent) which stains glycoproteins and polysaccharides red. The sections were counterstained with haemotoxylin to stain the highly eccentrically shaped nucleus (Fig 4.4).

Peripherally Located Ags in the PG Cells

Fig 4.5 shows the staining pattern observed with the MAb 4G2 (x 100). The Ag(s) against which the MAb is directed is concentrated in the connective tissue sheath. This type of pattern represents the most frequently observed Ag distribution from the fusion screen. This probably reflects the intact cellular nature of the immunogen and/or represents a dominant Ag.

Cytoplasmic Ags

Fig 4.6 is the staining pattern observed with the MAb 2H10 which selectively stains the cytoplasm (x 700). This photograph also

serves to demonstrate the irregular appearance of the nucleus which is unstained and therefore appears dark. Similarly the MAb 1C4 (Fig 4.7) also stains a cytoplasmically located Ag (x 700), but the staining shows a more heterogeneous distribution than that seen with 2H10.

The interpretation of some staining patterns using PG sections was sometimes difficult. However using intact, fixed, PG cells was not found to be a satisfactory alternative for cytoplasmic and nuclear stains because the pattern was masked by the extensive extracellular sheath. It was therefore important to make additional use of a different cell type to examine and interpret the distribution of Ags which were ubiquitous to all cells, that is, were not insect specific. The cells of choice were normal human fibroblasts because of their high cytoplasmic to nuclear ratio along with the ease with which they can be cultured in vitro on microscope slides. (The culture conditions are described elsewhere in this chapter).

An example of a cytoplasmic Ag which was difficult to interpret from PG sections but was elucidated using fibroblasts was the MAb 2H9 (Fig 4.8, x 700). It appears to be selective for a cytoskeletal Ag(s).

Nuclear Ag

Only a single cell line (1F8) was isolated from the fusion that secreted MAb directed against the PG nucleus. However the distribution of the nuclear Ag to which it was specific was not easily discernible in the two dimensional visualisation using PG tissue sections. Fibroblasts were therefore used as target tissue (Fig 4.9, x 700). The concentrated areas of staining within the

nucleus are very distinctive and whether this is a true representation of the physiological Ag distribution or the result of fixation is not known. However in unfixed fibroblasts, when viewed under an inverted microscope, similar, more numerous bodies in the nucleus can be seen (not shown). It is therefore possible that the distribution observed with 1F8 is of physiological relevance.

The preparation of normal human fibroblasts will now be discussed. Also the method of epithelial cell culture pertinent to the next section of this chapter will be detailed.

Preparation of Fibroblasts and Human Epithelial Cells for the Screening of MAbs

The normal human fibroblast cell line, FM730, and the human epithelial transformed cell line, HEP-2, were both generous gifts from the laboratory of Dr. D.W. Hough (University of Bath). The cell lines were cultured independently in 80 ml culture flasks (Nunc) in a 20 ml volume of Minimal Essential Eagles medium + 10% FCS and 4mM L-glutamine. (Incubator conditions are described in chapter II). The cells adhered to the culture flasks and so prior to manipulation they were trypsin treated to enable a cell suspension to be formed. To accomplish this the culture medium was drained from the flask and the cells washed in a calcium and magnesium free PBS (Flow) to remove all traces of FCS that would inhibit the trypsin. (The calcium and magnesium are both required for cell adhesion). Trypsin was then added in calcium and magnesium free PBS at a concentration of 0.05% (w/v) and incubated with the cells for 1 min. The volume of the trypsin solution was then reduced so that the cells were just covered and the flask incubated for 15 mins at $37^{o}C$. The vessel was then tapped briefly and the

cells examined under an inverted microscope. Following trypsin treatment the cells appeared spherical and were no longer adherent to the plastic. The cell suspension was then repeatedly pipetted to prevent clumping and 20 ml of culture medium added. The cell suspension was then placed in a sterile box (Nunc) containing sterile multispot microscope slides (Flow) so that the slides were just covered. The slides were incubated for 6-9 days with the cells, the medium was then discarded and the slides washed in 3 x 5 min changes of PBS. Fixation of the adherent cells was achieved by immersing the slides in ice cold acetone for 1 min and for a further 5 mins in fresh iced acetone. The slides were air dried and stored at $-40^{o}C$. The staining protocol was identical to that described for hydrated PG sections with the slides being warmed to room temperature prior to blocking. The slides permitted multiple test Abs to be applied to a single slide.

Investigation of Structural Ags

As previously mentioned, the vast majority of MAbs were directed against peripherally located Ags in the PG cells and in particular in the connective tissue sheath. From Fig 4.4 it can be seen that this area is rich in glycoproteins as would be anticipated for a region of connective tissue. The PG and the extracellular matrix are intimately associated and it seems likely that during development the PG secrete the necessary components for the formation of the connective tissue sheath. Alternatively they may do this in association with fibroblast-like haemocytes or other circulating cell types. The composition of the sheath is therefore an indication of the synthetic capability of the PG. An attempt was made to identify the possible nature of some of these Ags located peripherally around the cells and to see if these resembled vertebrate connective tissue components in terms of recognition

by the MAbs in question.

Collagen: A Putative Connective Tissue Component

A particularly important structural protein is collagen. Not only does it contribute to the form a tissue exhibits but it is also important in developmental processes. (For review see Bornstein and Sage, 1980). In Drosophila the expression of collagen genes is also a developmentally regulated process (Monson et al, 1982).

In the vertebrates the fibrillar and non-fibrillar collagens are genetically distinct. Types I, II and III are of the fibril forming type while the non-fibrillar collagen characteristically found in basement membranes is known as type IV (Miller and Gay, 1982; and reviewed by Bornstein and Sage, 1980). There is also a type V collagen, the classification of which has not been fully achieved, and is somewhat loosely termed 'cell associated' collagen (eg, Martinez-Hernandez et al, 1982; Bailey et al, 1979 and Haralson et al, 1980).

Collagens are produced from the proteolytic processing of pro-collagens, at least in the cases of types I, II and III. Collagens are comprised of three polypeptide α chains wound helically to form a rod-like structure. The α chains are synthesised on ribosomes which are membrane bound and enter the lumen of the endoplasmic reticulum (ER) as precursor molecules known as pro-α chains. Within the ER each pro-α chain associates with two other pro-α chains by hydrogen bonding to generate a triple stranded helical molecule. This pro-collagen molecule consists of a central collagen triple stranded helix with extension peptides at each end of the three constituent chains. During secretion of collagen from the cell the extension peptides are cleaved off by pro-collagen peptidases. The resulting collagen

is secreted into the extracellular matrix and associates with other collagen molecules to form large fibrils. These tend to form over the secreting cell and, under the cells control, a network of fibrils is laid down. The network is strengthened by covalent bond formation between, and within, collagen molecules. Type IV collagen does not undergo this proteolytic processing although pro-collagen does undergo some modification when it is secreted from the cells and incorporates into the connective tissue matrix. Purified type IV collagen is also slightly modified during its extraction as some end regions break down and so a distinction between collagen IV and its pro-collagen is valid (see review by Bornstein and Sage, 1980).

Invertebrate Collagens

Collagens from different vertebrate species are all similar, with little interspecific variation (Adams, 1978). The invertebrates, however, consist of a very diverse range of organisms, by comparison to the vertebrates, and these animals possess equally diverse functional and structural characteristics. It is therefore unfortunate that the majority of studies to date have been concerned with vertebrate collagens with only sparse attention paid to these structural proteins in invertebrates.

Collagens have been defined on the basis of their x-ray diffraction patterns and ultrastructural appearance, but also by amino acid analysis that has revealed every third amino acid residue to be glycine, a feature which is essential for triple helix formation. Typically, elevated levels of hydroxyproline and proline are present. However not all collagens fit this basic pattern and some collagens have been found to contain no measurable proline (Spiro et al, 1971). Variability in bonding patterns at the ultrastructural level also occur (reviewed by Adams, 1978).

Collagens have been isolated from all metozoa and are ubiquitous structural proteins despite some minor variability. The most frequently studied invertebrate collagens have been derived from nematodes, annelids and coelenterates (see Adams, 1978) because their cuticles are rich in collagen.

Vertebrate collagens, with the exception of type I all consist of three identical peptide chains. Some invertebrate collagens have been studied and also found to consist of three α chains, with a total mwt of 300KD, which is identical to that of the vertebrate collagens. (For review see Adams, 1978). However the collagen derived from crab and lobster both resemble type I collagen in vertebrates by virtue of having the sub unit formula of $[(\alpha 1)\ 2\alpha 2]$, see Table 4.1. Whereas vertebrate interstitial (non-basement membrane) collagens are all virtually identical in amino acid composition, and when the amino acids are sequenced show extensive homology between the same collagen type in different species, the invertebrates show a variety of amino acid compositions. As yet no systematic sequencing of invertebrate collagens has been reported. However, what is well documented in vertebrates and invertebrates is the variation of collagen types within a single organism. This intraspecies differential collagen expression has been reported in the vertebrates (Miller, 1976) and probably reflects tissue differentiation and differing functional requirements of tissues. Similar findings have resulted from studies on Ascaris and Lumbricus (Fujimoto and Adams, 1964).

Collagens are frequently found in association with carbohydrates. For instance interstitial collagens in vertebrates are glycoproteins with some hydroxylysyl residues substituted with galactose or glucosylgalactose (Miller, 1976). Vertebrate basement membrane collagen contains glycosylated hydroxylysyl residues and

other sugars can also be associated with collagen (Kefalides, 1975). Generally, in the handful of species studied to date, invertebrate collagens contain more saccharide units by comparison with vertebrate interstitial collagens. Within the vertebrates, type IV collagen has more saccharide associations than vertebrate interstitial collagens.

In conclusion the collagens show considerable conservation of structure throughout the metozoa. However modifications of collagens do occur in different tissues and animal groups which probably reflects differential functional requirements.

Insect Collagens

Collagen in insects exists in both fibrillar and basement membrane forms providing internal support to body organs and also beneath the epidermis (see review by Ashhurst, 1968). In contrast to the situation in eg, Ascaris where collagens are important structural components of the exoskeleton, in the arthropod cuticle collagen is entirely absent and this role is played by the polysaccharide chitin.

In the hemimetabolous insects, such as the locusts and cockroaches, the collagen fibrils are relatively large and distinctly banded when examined ultrastructurally; such banding is considered to reflect gross sequence information (Adams, 1978). In contrast some holometabolous insect groups such as the moths and flies have smaller fibrils with indistinct banding (Locke and Huie, 1972). The fact that very few tissues in insects serve as sources of large quantities of collagens has certainly been a factor in slowing the rate of progress in this field of study. This has also led to a very restricted range of species being examined. However there have been a few isolated studies such as the study of collagen

in Locusta migratoria which has a relatively rich source of collagen in the tissue which surrounds the male ejaculatory duct in adults (Ashurst and Bailey, 1980). The collagen is secreted by fibroblasts (Ashurst and Bailey, 1980). On the basis of ultrastructure the locust collagen was found to closely resemble mammalian type I collagen (Ashurst and Bailey, 1980). On biochemical analysis the α chains were found to resemble vertebrate $1\alpha(1)$ chains but contained relatively more hydroxylysine residues. The locust collagen was found to consist of three identical $\alpha 1$ units. Low levels of type IV-like collagen were also present (Ashurst and Bailey, 1980). This data is in keeping with the other invertebrate studies in which fibrous collagens were found to resemble vertebrate type I collagen.

Perhaps of particular relevance to the study in this thesis is the characterisation of collagen in a holometabolous species, Tenebrio molitor (Francois, 1985). The mesenteric sheath derived collagen was extracted and found to be made up of three identical α-chains with amino acid analysis revealing one third of the residues to be glycine. The collagen formed fibrils and was very similar ultrastructurally to type I vertebrate collagen.

The collagen of another holometabolous species, the fruit fly, Drosophila melanogaster, has been investigated using the methods of molecular genetics. Because of the obvious importance of such a study in an otherwise sparsely investigated field it is worthy of some discussion. Monson et al (1982) probed a Drosophila genomic library using a chicken $\alpha 2(1)$ cDNA clone as hydridisation probe. The gene they located coded for 469 amino acids and unlike vertebrate fibrillar collagen genes did not have an uninterrupted sequence. This was thought to imply that the gene may code for a non-fibrillar collagen. The fact that the probe would hybridise

with a non-fibrillar collagen at all is probably because of the repetitious characteristic of collagen coding sequences coupled with the unusual codon usage in collagen genes. The Drosophila gene consists of two large coding sequences, the product of which has the typical amino acid composition of triple-helix forming peptides with every third amino acid being glycine. From studies on vertebrate fibrillar collagen genes it has been thought that the genes were derived from the amplification of a 54 base pair 'primordial' unit but interestingly no evidence was found in the Drosophila study to suggest that this was also the case in this invertebrate. Either this model does not apply, or the Drosophila gene has evolved in such a way that the underlying pattern has become masked. At present there is no information on the genomic organisation of invertebrate fibrous collagens or on vertebrate non-fibrous collagens. There is therefore no evidence as to whether the Drosophila gene sequence simply reflects differences between the invertebrates and vertebrates or differences between fibrillar and non-fibrillar collagens. It also remains a possibility that the Drosophila gene codes for a novel collagen type. Expression of mRNA that hybridises with a probe made from this gene is heightened during the first and second larval instars when cuticle and basement membrane formation is most active, although there remains the possibility that other collagen types are synthesised in epidermal cells at other developmental stages.

The molecular status of basement membrane collagens in insects remains unclear and its relation to vertebrate type IV collagen is not known. The present state of knowledge of the molecular composition of vertebrate and invertebrate collagens are summarised in Table 4.1.

Antigenicity of Collagens

The majority of polyclonal Abs have been raised against vertebrate type I pro-collagen or type I collagen immunogens (reviewed by Bornstein and Sage, 1980). There is evidence of shared sequences and/or three-dimensional conformation between collagen types which has resulted in extensive affinity purification being required to eliminate or reduce cross reactivity between different collagen types (Bornstein and Sage, 1980). There have, to date, been no reports of polyclonal or monoclonal antisera raised against invertebrate derived collagens despite the fact that these would provide useful tools for the study of invertebrate collagens. The data reported in this chapter is therefore of considerable relevance as it details the partial characterisation of collagen-like Ags derived from Manduca PG using specific MAbs, and attempts to relate the results to what is known of vertebrate collagens.

Detection of Collagen-like Ags in Manduca PG

All the MAbs that had been screened against PG tissue sections (see earlier this chapter) and found to selectively stain the connective tissue sheath or cytoplasm were re-screened in an ELISA using commercially available vertebrate collagens as coating Ags. For this assay fibrillar type I collagen was selected because it is the most abundant collagen type, at least in vertebrates where 90% of the collagen is type I. Also the non-fibrillar type IV collagen was used in parallel with type I to gain insight into whether or not collagen or collagen-like molecules occur in Manduca and if so to what structural form are they most similar on the basis of Ab recognition.

Collagen ELISA Methodology

Flexible PVC 96 well activated ELISA plates (Flow) were coated with 100 ul per well of a 5 ug per ml collagen solution in PBS. Two types of collagen were used. Rat tail collagen (Sigma) was used as a source of collagen type I. This occurs in skin and tendon and is acid soluble. To coat the plate this collagen was initially solubilised in 0.5 ml of 2M HCl, the concentration of the solution then being adjusted to 5 ug per ml while the pH was adjusted to 6.5 using KOH. The second type of collagen, type IV, was derived from a human placental basement membrane preparation (Sigma). This type of collagen is soluble at neutral pH and so was dissolved directly in PBS (pH 7.2) to give a final concentration of 5 ug per ml. The ELISA protocol was essentially identical to that described in chapter III. Briefly, the plates were coated overnight at 4°C prior to the assay the following day. The plates were wrapped in 'clingfilm' to prevent evaporation. The coating solution was discarded and 100 ul of a 1% (w/v) casein solution in PBS was added to each well and incubated at 37°C for 30 mins. After blocking the plates were washed with 3 x 5 min changes of PBS/Tween 20 (0.05%) and then culture supernatants from selected cell lines (see above) were incubated for 2 hours at 37°C. The washing step was repeated and 100 ul of 1:1000 (v/v) rabbit anti-mouse alkaline phosphatase conjugate (Sigma) in PBS was added to each well and incubated for 1 hour at 37°C. The plate was washed (see above). The phosphatase substrate 'Sigma 104' (Sigma) was dissolved in a 10% (w/v) diethanolamine buffer pH 9.6 at a concentration of 1 mg per ml and 100 ul placed in each well. After 40 mins the plates were read using a Titertek ELISA plate reader using a filter of 405 nm to give absorbance values for each well.

Results and Discussion

94 culture supernatants were tested in the ELISA to investigate whether or not any of the MAbs would bind to types I and IV vertebrate collagens under the ELISA conditions used. The assays were performed in parallel and in duplicate. Background was assessed by the inclusion of an 'irrelevant' MAb. Wells were designated as 'positive' if their absorbance value was at least 10 x greater than that of the background wells.

The results are summarised in Table 4.2. Six cell lines were positive when assayed against type I vertebrate collagen. Only a single cell line, 1C8, was positive against type IV collagen but interestingly it was also positive against type I collagen. Of the five cell lines which secreted MAb that bound only type I collagen, all were used to stain PG sections, human fibroblasts and human, HEP-2, epithelial cells. They all gave identical staining patterns and so only 4B6 staining is demonstrated photographically. For a list of figures see Table 4.2.

From the ELISA data summarised in Table 4.2 it can be seen that collagen or collagen-like Ags occur in the PG of Manduca. Of the six MAbs that were positive in the assay all bound to ELISA wells coated with type I collagen (fibrillar) but interestingly one of these MAbs, 1C8, also bound to type IV collagen in the ELISA system. The fact that 1C8 will recognise both collagen types in the assay reflects that the two molecules share a common antigenic determinant which must differ from the antigenic site recognised by the five MAbs that failed to bind type IV collagen in the assay. From this small library of MAbs it was possible to use the discriminatory properties of the MAbs to see if any additional insight into the original Ags was possible by staining cells

with the Abs using an indirect immunofluoresence technique. The cells chosen were the PGs which had formed the original immunogen, fibrillar collagen secreting vertebrate fibroblasts and basement membrane collagen secreting vertebrate epithelial cells. It had been initially anticipated that collagen could perhaps form a significant component of the PG extracellular matrix but interestingly the type of staining pattern observed with 4G2 (Fig 4.5) did not occur with the collagen binding MAbs. Instead weak, diffuse staining by the MAbs was observed in the peripheral cytoplasm and extracellular matrix. The staining pattern exhibited by 1C8 was typical, see Fig 4.10. Perhaps this reflects the relative abundance of the Ag(s) or simply reflects the avidity of the MAb or the result of fixation. However it could also be possible that the weak, peripheral cytoplasmic stain observed is the result of mvs containing proteins destined for incorporation into the basal lamella. If this was the case the vesicles would be anticipated to contain collagen-like materials. It was therefore of interest to investigate the staining capability of the MAbs against vertebrate collagen secreting cell lines. This would also provide insight into whether pro-collagens were recognised by the MAbs as both vertebrate cell types used should contain intracellular pro-collagens. The staining patterns observed with the five type I positive MAbs were identical and so a representative MAb, 4B6, is depicted photographically.

The patterns of staining by both 4B6 (Fig 4.13, Fig 4.14) and 1C8 (Fig 4.11, Fig 4.12) against both fibroblasts and HEP-2 cells was intracellular in nature. Both MAbs staining fibroblasts exhibit a characteristically perinuclear ring of stain but 1C8 (Fig 4.11) shows considerably more cytoplasmic staining in the form of a network-like pattern extending into the furthest reaches of the cell

cytoplasm by contrast to 4B6 (Fig 4.13). It does not appear to be the result of differential affinities by the MAbs for the intracellular Ag(s) but 1C8 (Fig 4.11) recognises Ag(s) with a much wider distribution within the cell. As both MAbs exhibit the same perinuclear pattern the 1C8 pattern (Fig 4.11) can be envisaged to be a common Ag(s) to which 4B6 will also bind but superimposed on this is the Ag distribution uniquely recognised by 1C8. Extracellular staining by both MAbs was very restricted and limited only to regions on the glass slide where cytoplasmic extensions of the fibroblasts had been withdrawn during culture (see arrow Fig 4.13). This perhaps indicates that the protein matrix used for cell adhesion contains Ag(s) recognised by the two Abs. It also serves to demonstrate that fibroblasts in culture secrete very little collagen (probably type I) under the culture conditions used. This is a reasonable assumption because it has already been demonstrated in the ELISA (Table 4.2) that both MAbs will bind type I collagen. It appears unlikely that there is such a difference in the assay conditions to the cultured cells that the Abs would fail to recognise the secreted collagen although this does remain a possibility.

The staining patterns observed using human epithelial cells (HEP-2) as the target tissue are masked to some extent because of the lower cytoplasmic to nuclear ratio by comparison to fibroblasts. However, essentially the same intracellular staining pattern can be seen with 1C8 (Fig 4.12) and 4B6 (Fig 4.14). 1C8 perhaps shows a more clearly defined staining pattern which could be due to higher avidity or differential Ag recognition by comparison to 4B6. What is interesting is that there is extensive intracellular staining of what are typically type IV collagen secreting cells, at least in their untransformed state. Normal

epithelial cells secrete type IV collagen for basal lamella formation. The staining observed here is intracellular in nature, again indicating the Abs might be recognising a pro-collagen-like Ag or an intracellular collagen-like Ag. The fact that 4B6, which failed to recognise purified type IV collagen in the ELISA, stains type IV secreting cells may indicate that it is another protein being recognised by the MAb in the cell, perhaps some sort of intermediate filament protein which shares a determinant with type I collagen? One way of testing this would be to stain non-collagenous cell types to see if a similar staining pattern is observed. Alternatively, this could merely reflect that 4B6 recognised a region of collagen type IV which is significantly modified during collagen extraction such that the MAb fails to recognise the pure preparation in the ELISA.

With respect to the tissue of interest, namely the PG, what can be deduced? There is undoubtedly an Ag(s) with a determinant(s) that is shared in common with vertebrate collagens of types I and IV. This could reflect homology between the Ag(s) and vertebrate collagens implying the Ag is a structural protein with collagen-like structure and function or that the shared determinants are due to unrelated cross reactivity between functionally related molecules.

The MAbs directed against the PG also show some homology or unrelated cross reactivity with intracellular components of both human fibroblasts and epithelial cells as well as the matrix which facilitates cell adhesion. It seems likely that by virtue of the fact that the two MAbs recognised to differing extents the two vertebrate collagens used in the ELISA, that they could also recognise pro-collagens which are at least, in part, structurally very similar to the final secreted collagens. This could explain the fibroblast staining pattern shown by the two MAbs, although it

does not explain the extended pattern of staining exhibited by 1C8. This latter observation implies the presence of another Ag in the cell that shares the antigenic determinant recognised by 1C8. The pattern superficially resembles intermediate filament distribution and certainly this class of intracellular structural protein could conceivably show functional cross reactivity although biochemical analysis of the Ags would be required to demonstrate this conclusively. A similar rationale could explain the patterns observed with HEP-2 cells.

The MAbs outlined in this chapter are potentially useful tools for the study of structural components in the extracellular matrix which forms such an important connective tissue sheath around the cells. The MAbs could be used for isolating the Ags in the PG, fibroblasts and HEP-2 cells and, by biochemical analysis, the extent of the similarity or difference between these Ags could be examined and compared to commercially available pro-collagen preparations. This would help to answer some of the questions discussed above. A more precise cellular and extracellular localisation of the Ags in the PG could be achieved at the ultrastructural level using immunogold staining in conjunction with EM.

Future of Collagen Studies in Insects

Biochemical characterisation and ultrastructural techniques have constituted the major approaches in the study of insect collagens to date, the exception being the investigation of non-fibrous collagen genes in *Drosophila* (Monson *et al*, 1982). The extent of knowledge on the genetics of *Drosophila* make it a likely candidate for future attempts to study fibrillar collagens and this will give a valuable insight into the relationship between vertebrate and invertebrate collagens. None of the studies of insect collagens have utilised

specific Abs as tools for the investigation of these structural proteins. Abs would enable affinity purification of the Ags and MAbs to connective tissue preparations could be invaluable in probing for as yet uncharacterised insect structural proteins. MAbs could also be a means of discriminating more accurately between different collagen types and their pro-collagens which has proved difficult with polyclonal antibodies (Bornstein and Sage, 1980). The use of MAb could also be very useful in the study of invertebrate collagens that are indistinctly banded when examined at the ultrastructural level. The advent of immunogold preparations for EM should greatly facilitate the study of, eg, Dipteran and Lepidopteran fibrous collagens.

The MAbs described in this chapter have many potential applications. It would be of interest to study the tissue distribution of the Ags in the insect and to investigate any developmental regulation of the collagen-like Ags. This is of particular interest in Manduca because of the body of developmental information known. Some insight could also be gained by studying the possible distribution of these Ags in insect tissues from other species known to be rich in collagen eg, locust ejaculatory duct (Ashurst and Bailey, 1980). Full biochemical analysis of the Ags would be very useful.

In conclusion there is evidence of collagen-like structural molecules in the PG extracellular matrix and peripheral cytoplasm from the MAb studies described in this chapter. Some indication of the possible nature of these Ags is given and some discussion as to the implications and future areas of study.

Table 4.1a Summary of Vertebrate Collagen Types and their Molecular Composition.
(After Bornstein and Sage, 1980)

Table 4.1b Summary of Some Invertebrate Collagens.

References:

1. Adams (1978)
2. Ashhurst and Bailey (1980)
3. Francois (1985)
4. Monson et al (1982)

Type	Chains	Molecular Composition	Tissue
I	$\alpha 1, \alpha 2$	$[\alpha 1(I)]2\alpha 2(I)$	Skin
II	$\alpha 1$	$[\alpha 1(II)]3$	Cartilage
III	$\alpha 1$	$[\alpha 1(III)]3$	Foetal Skin
IV	$\alpha 1 \beta 2$	Not Known	Placenta
V	$\alpha 1 \beta 2 \beta 3$	Not Known	Amnion and Chorion: Placenta

Type	Chains	Molecular Composition	Animal
I-like	$\alpha 1 \beta 2$	$[(\alpha 1)2\alpha 2]$	Lobster[1] Echinodern[1] Nereis[1]
I-like	$\alpha 1$	$(\alpha 1)3$	Locust[2] Tenebrio[3] Actinia[1]
IV-like	Not Known	Not Known	Locust[2]
IV-like or or novel type	Not Known	Not Known	Drosophila[4]
Differs from Vertebrate Collagens	Not Known	Not Known	Ascaris[1]

Table 4.2 Summary of ELISA Data and Indirect Immunofluorescence for the Six 'Anti-Collagen' MAbs.

Ascitic fluid was produced from cell lines 1C8 and 4B6 and both were of the IgM isotype.

+ = at least 10 x greater absorbance value than background.

- = not significantly different from background.

Background = 'irrelevant' MAb (isotype matched).

	Collagen ELISA		Indirect Immunofluorescence					
Cell Line	Type I	Type IV	PG	Fig	Fibroblasts	Fig	HEP2	Fig
1C8	+	+	Weak stain in periphery and extracellular matrix	4.10	Extensive intra-cellular staining. Perinuclear ring and cytoplasmic network	4.11	Perinuclear ring and cytoplasmic network	4.12
4B6	+	-	"		Stain concentrated in perinuclear ring Some weak general cytoplasmic staining	4.13	Perinuclear ring and some general cytoplasmic staining	4.14
1D2	+	-	"		"		"	
4B5	+	-	"		"		"	
1G11	+	-	"		"		"	
4B9	+	-	"		"		"	

Fig 4.1 SEM of the PG of Manduca, W + 2 in the fifth instar caterpillar. The body of gland cells (P) are associated with the main tracheal trunk (T) which opens into the prothoracic spiracle. Strands of cells can be seen to extend from the prothorax towards the head. The cells are encased in an extracellular connective tissue sheath.

The scale bar represents 100 microns.

Fig 4.2 TEM of the peripheral channel system of a W + 2 PG cell of Manduca sexta. BL = basal lamina; PCS = peripheral channel system; m = mitochondrion; mvs = multivesicular sac (x 33,000)


15KV
2682
100.0U
BATHU

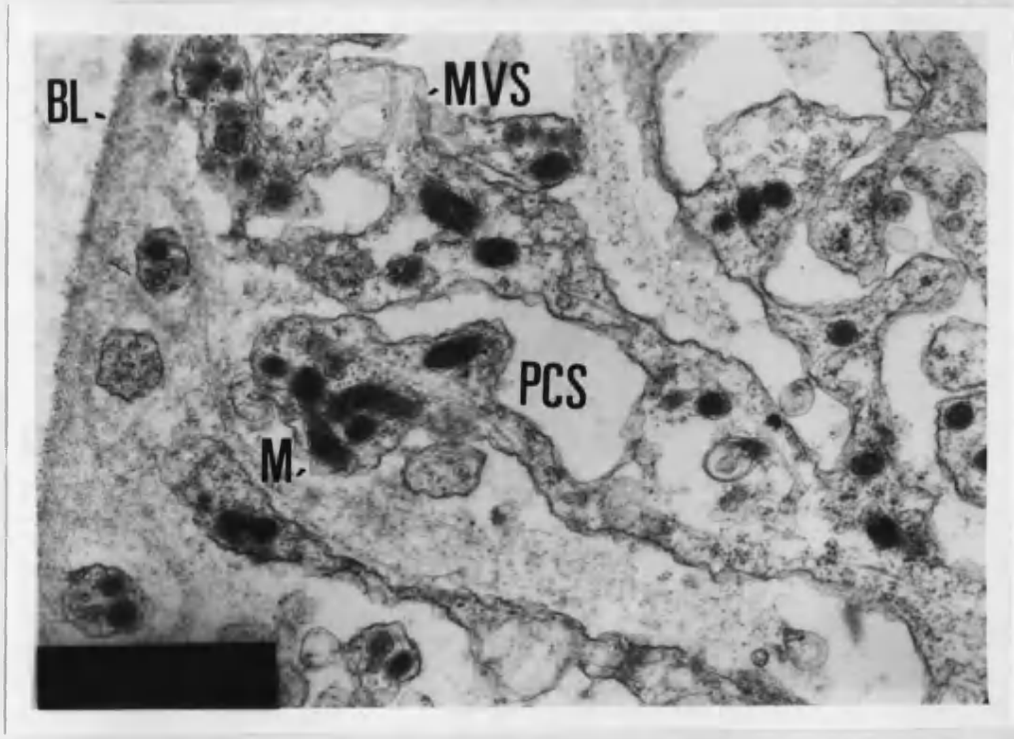
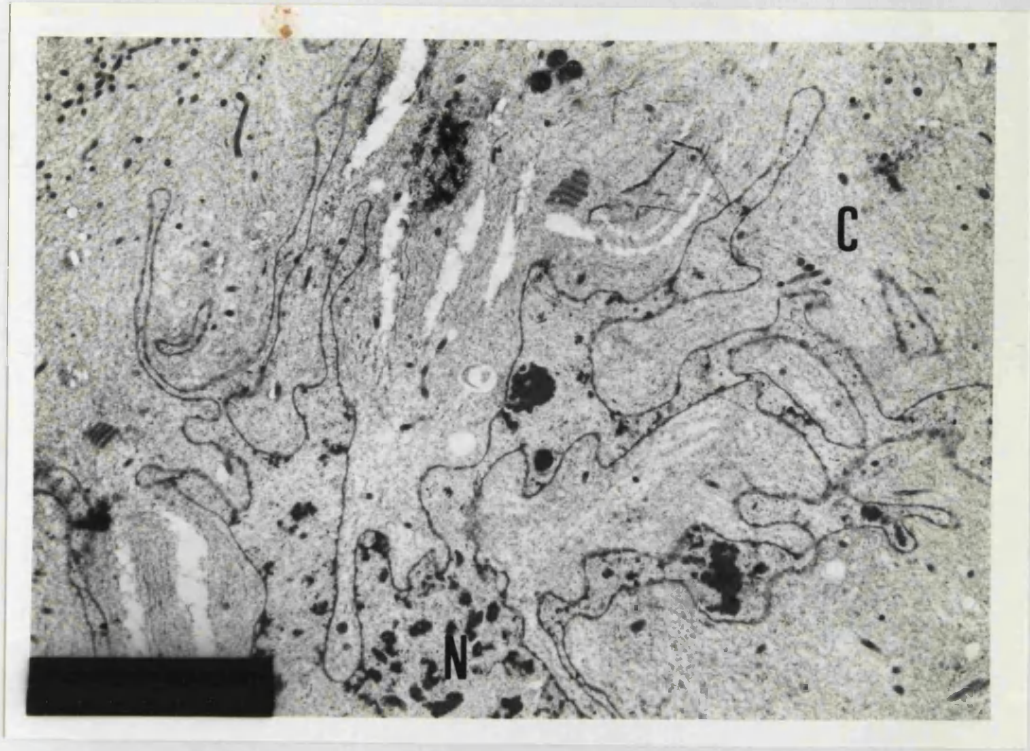

BL
MVS
PCS
M

Fig 4.3 TEM depicting part of the nucleus (N) and surrounding cytoplasm (C) of a W + 2 PG cell of Manduca (x 13,000)

Fig 4.4 Two PG cells stained with PAS and counterstained with haematoxylin to illustrate the basic structural composition of W + 2 PG cells. The extensive invagination of the plasma membrane, characteristic of PG cells during ecdysone secretion, is clearly visible. PCS = peripheral channel system; n = nucleus. The nucleus is blue, cytoplasm pale pink and connective tissue red (x 200).


C
N

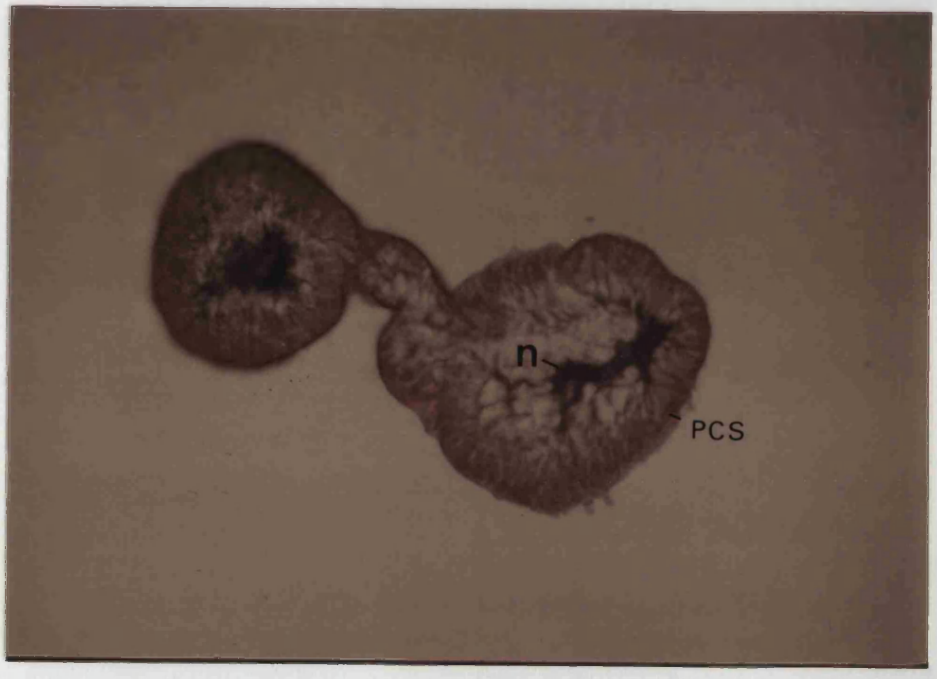

n
PCS

Fig 4.5 MAb, 4G2, staining of PG (W + 2) by indirect immunofluorescence (FITC). The staining is concentrated in the connective tissue sheath (x 100).

Fig 4.6 MAb, 2H10, staining PG (W + 2) by indirect immunofluorescence (FITC). The staining pattern is selectively cytoplasmic (x 700).

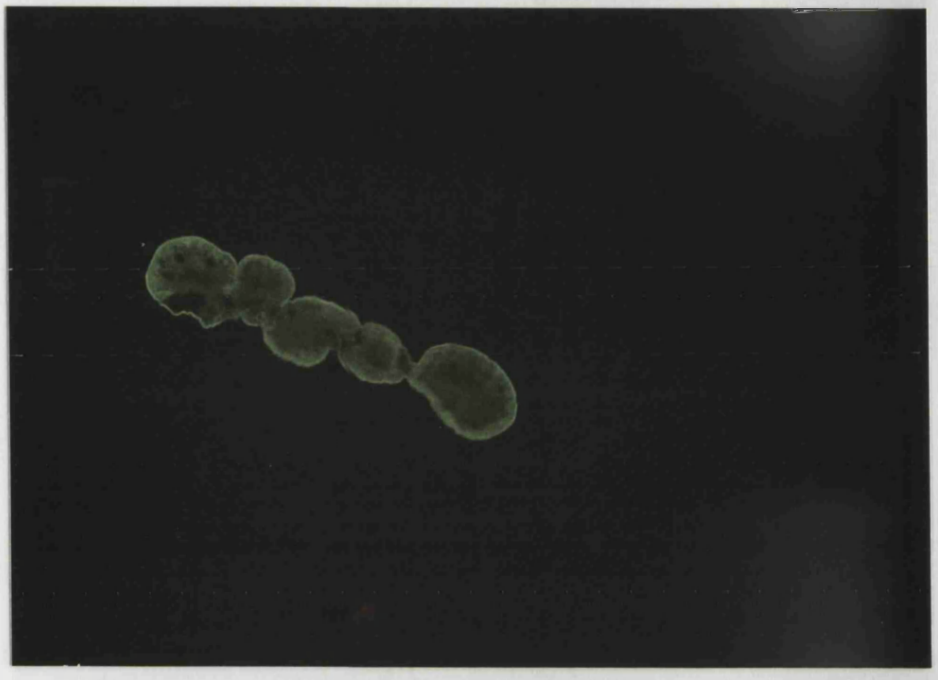
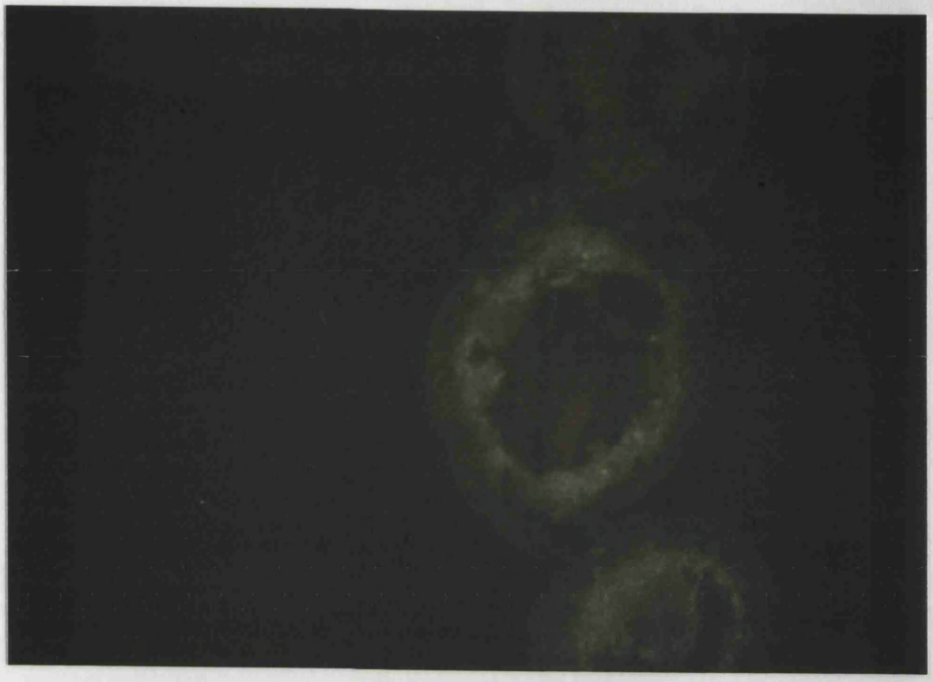


Fig 4.7 MAb, 1C4, staining PG (W + 2) by indirect immunofluorescence (FITC). The staining pattern is selectively cytoplasmic although more heterogeneously distributed than 2H10 (x 700).

Fig 4.8 MAb, 2H9, staining normal human fibroblasts by indirect immunofluorescence (FITC). The pattern appears to be cytoskeletal in nature (x 700).



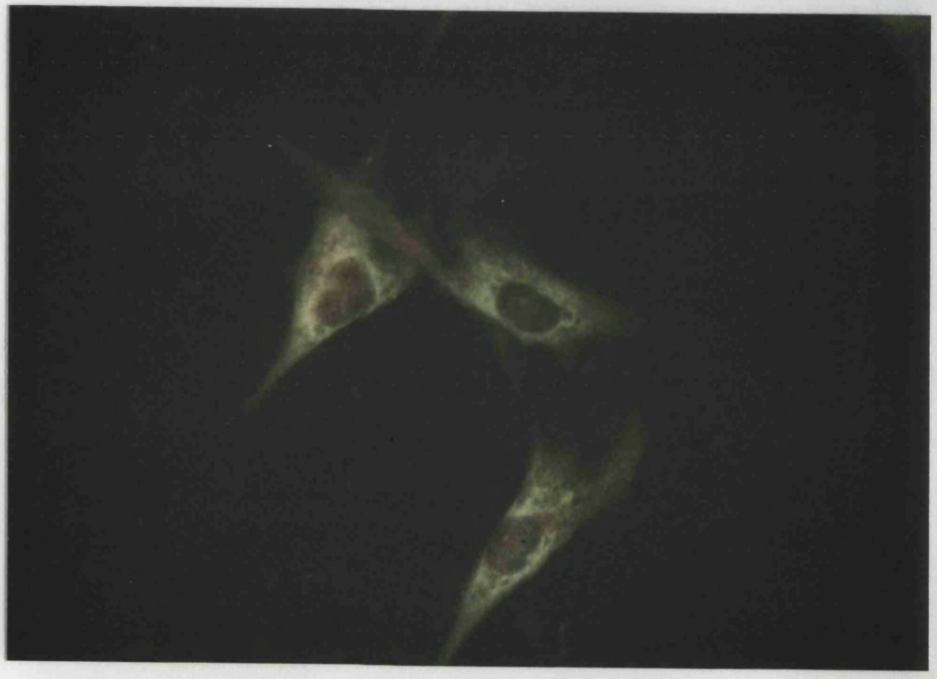


Fig 4.9 MAb, 1F6, staining normal human fibroblasts by indirect immunofluorescence (FITC). The antigen recognised by the MAb is located in the nucleus and appear as bright 'spots' of staining (x 700).

Fig 4.10 MAb, 1C8, staining of PG cells (W + 2) of Manduca by indirect immunofluorescence (FITC). Weak staining is observed in the peripheral cytoplasm and extracellular sheath (x 700).

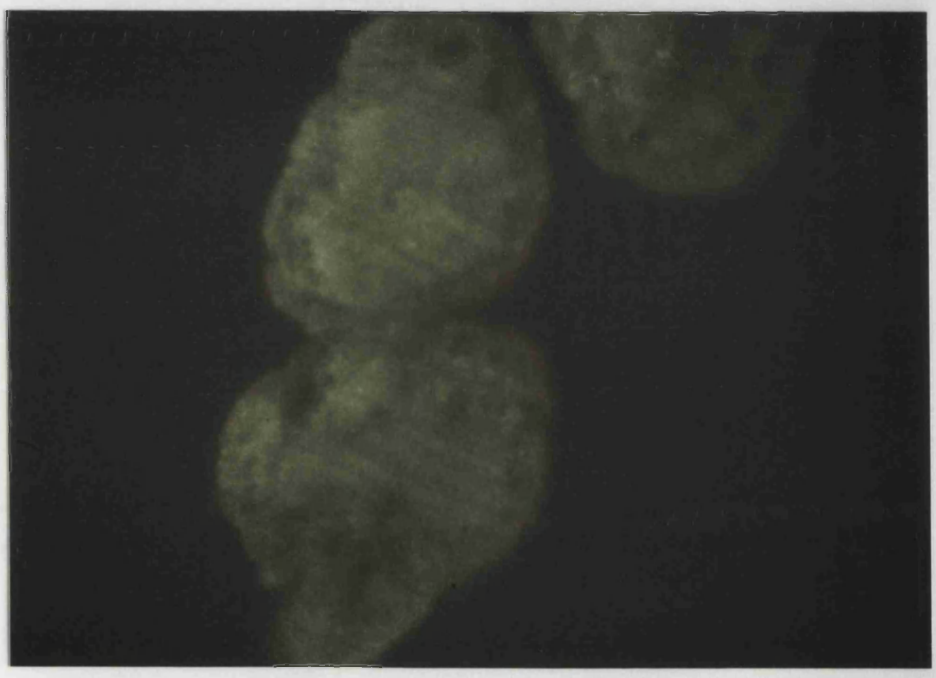
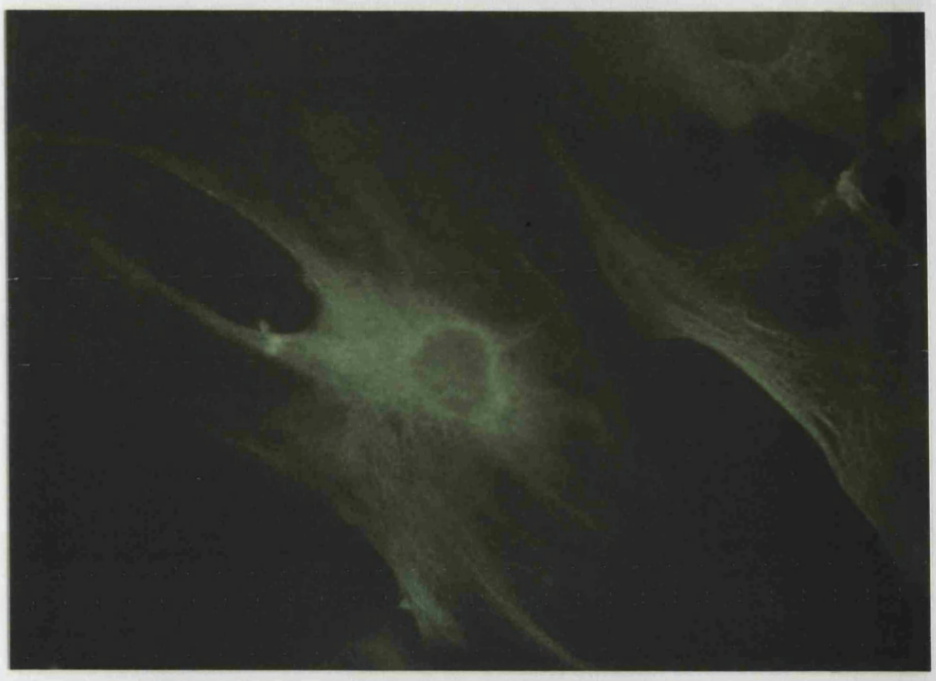


Fig 4.11 MAb, 1C8, staining normal human fibroblasts by indirect immunofluorescence (FITC). The staining is concentrated around the nucleus to form a perinuclear ring and there is extensive cytoplasmic staining that extends into the cytoplasmic extensions (x 700).

Fig 4.12 MAb, 1C8, staining HEP-2 cells by indirect immunofluorescence (FITC). The staining pattern comprises a perinuclear ring and cytoplasmic network (x 700).



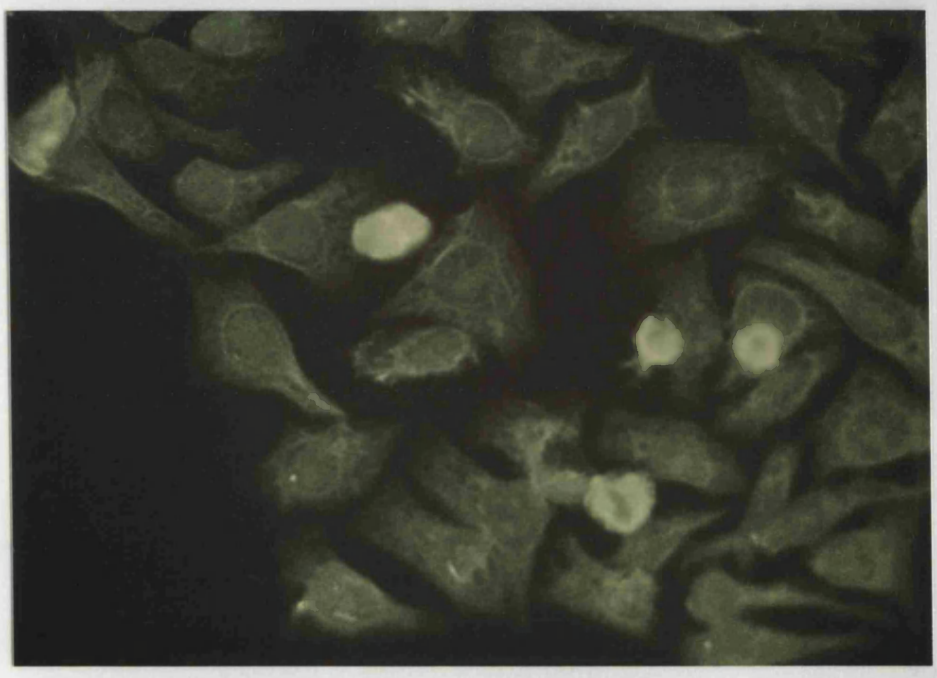
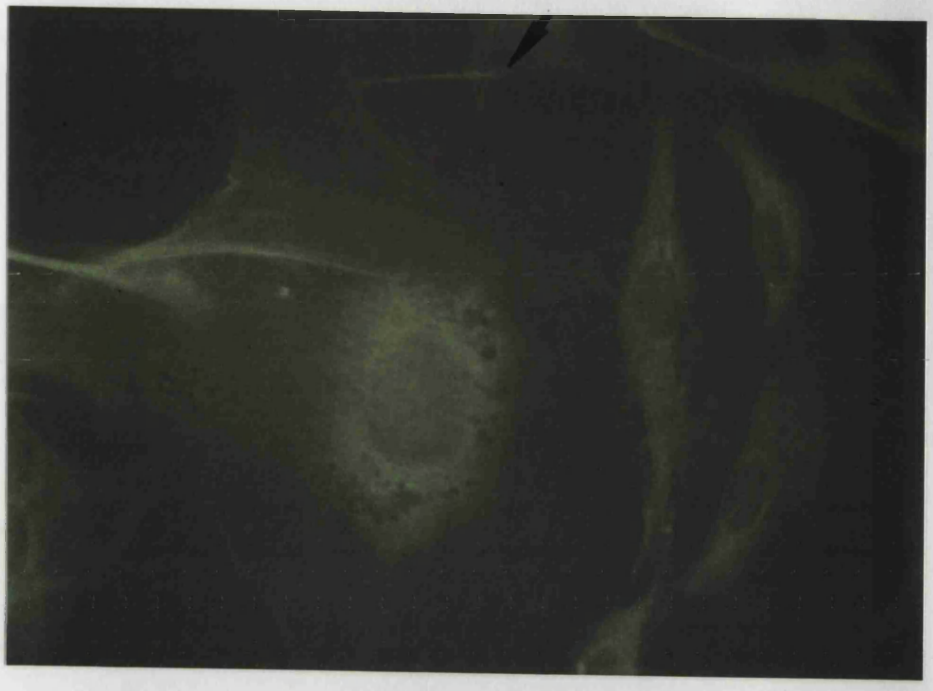
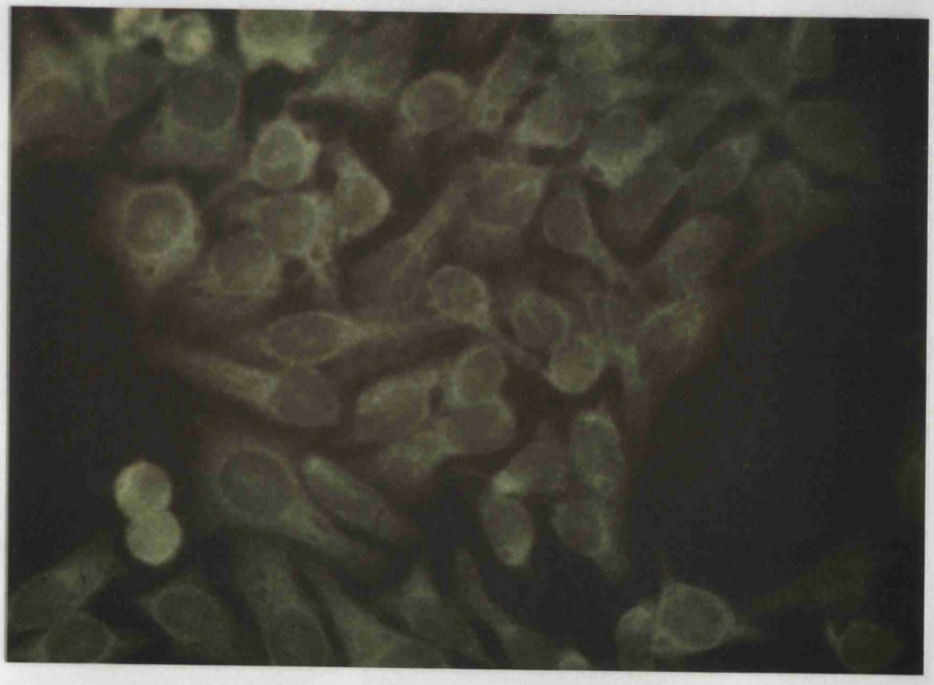


Fig 4.13 MAb, 4B6, staining human fibroblasts by indirect immunofluorescence (FITC). The patterns shown staining around the nucleus and restricted cytoplasmic staining.
Arrow pinpoints extracellular stain probably attributable to an area of former cell adhesions (x 700).

Fig 4.14 MAb, 4B6, staining of HEP-2 cells by indirect immunofluorescence (FITC). The staining is concentrated around the nucleus but also some cytoplasmic staining can be seen (x 700).





Chapter V: An Investigation and Partial Characterisation of the G6 Ag(s)

Introduction

As previously outlined (see chapter IV) MAbs were raised against an immunogen of crude PGs from W + 2 *Manduca sexta* larvae. This chapter describes the screening of these MAbs against free ecdysone, the isolation of the MAb (G6) which bound the free steroid, the subsequent partial characterisation of the G6 Ag in the PG, and the study of its tissue and developmental distribution.

The Screening of MAbs

The work of King *et al* (1974) established that the PG of *Manduca* contain no detectable free ecdysone when analysed by RIA, the supposition being that the glands secrete the steroid as it is synthesised and do not act as storage sites for ecdysone. It therefore appeared unlikely that any MAbs would have been generated from the fusion (described in chapter IV) that would bind free ecdysone, particularly considering the low mwt of the molecule and the apparently undetectable levels of the steroid in the glands (see above). However it was of interest to test this premise and therefore all the MAbs generated from the fusion were assayed using the ELISA described in chapter III, using free ecdysone or a 20-HE-BSA conjugate as the coating Ag (see chapter IV for detailed protocol). The MAbs were screened in parallel against casein and BSA coated wells to detect MAbs which cross reacted with the blocking agents.

Results of The Screen

Of the wells that showed hybridoma growth 2.5% were found to be positive in the ELISA using 20-HE-BSA or ecdysone coated wells. The two cell lines which gave the highest absorbance values (greater than 10 x background) in the ELISA were cloned and ascitic fluid produced (see chapter II). The remaining positive cell lines were stored under liquid nitrogen.

The ascitic fluid was tested against ecdysone coated wells in the ELISA to examine the extent of intra-assay variability attributable to each cell line. The data and characteristics of the two MAbs, IE9 and G6 are summarised in Table 5.1. Because of the low variability between intra-assay replicates attributable to the G6 MAb, this Ab was used to characterise the Ag(s) in the PG which had elicited the immune response of the G6 cell line.

Testing of the G6 MAb in an RIA Binding Assay

The data outlined in chapter III indicates that the affinity of an Ab must be relatively high if the MAb is to precipitate radiolabelled ecdysone under RIA conditions.

The MAb, G6, would bind ecdysone under ELISA conditions and so it was tested in the RIA binding assay (see chapter III) to investigate its ability to precipitate radiolabelled ecdysone. The G6 ascitic fluid was diluted using BB + 1% BSA to give a final dilution range of 1:50 to 1:2000. 100 ul of each dilution was added to 100 ul of BB + 1% BSA containing approximately 4000 cpm ^{3}H-ecdysone. Following incubation overnight at $4^{o}C$ the MAb was precipitated using SAS and counted in an LKB liquid scintillation counter (for complete methodology see chapter III).

Results

There was no significant difference between the G6 precipitated samples (x = 16.4 cpm ± 1.2, n = 12) and the background (x = 15.9 cpm ± 1.1, n = 12) expressed as the mean ± SE.

The failure of the G6 MAb to precipitate ^{3}H-ecdysone probably reflects low affinity for the steroid or alternatively was due to the particular separation technique used. Assay specificity is a relatively common phenomenon with some MAbs (Haaijman et al, 1984). The failure of the G6 Ab to precipitate the steroid was not due to its failure to bind to the tritiated form of the hormone because when 2 ug per ml ^{3}H-ecdysone was used as coating Ag under ELISA conditions the G6 MAb bound to the steroid and the absorbance readings did not differ significantly from those obtained from wells coated with 2 ug per ml of unlabelled ecdysone. To investigate the nature of the G6 Ag(s) further, PG extracts were used to inhibit the binding of the G6 MAb to ecdysone coated wells in the ELISA system described above.

Immunoreactivity in PG Extracts

Ten pairs of W + 2 PG were homogenised in 100 ul PBS (pH 7.2, 0.15M) in a tissue grinder (Bolab) and spun in a microcentrifuge (Eppendorf 5412) for 5 mins. The supernatant was removed and 20 ul (equivalent to two pairs of glands) was serially diluted in PBS in duplicate. 10 ul of extract, at various dilutions corresponding to amounts of tissue ranging from 1 gland to 0.0001 gland, were then mixed with 100 ul of G6 ascites (1:200 in PBS) and pre-incubated overnight at 4^{o}C in siliconised glass test tubes. Control samples were prepared using 'irrelevant' MAb ascites, at a comparable protein concentration, along with G6 aliquots pre-incubated with PBS

(10 ul). Following pre-incubation the samples were assayed in the ELISA (see chapter III) using 2 ug per ml ecdysone in PBS as coating solution and 1% casein in PBS as blocking agent.

Results

Fig 5.1 indicates that an Ag(s) in the PG extract could inhibit binding of the G6 MAb to ecdysone, under ELISA conditions, within the range of 0.5 to 0.001 of a PG. At PG concentrations greater than the equivalent of 0.5 of a gland, enhanced binding was seen indicating either that at this concentration the G6 Ag(s) binds to the plastic surface of the well (despite blocking) or alternatively that the G6 Ag(s) binds to the ecdysone coating the well at this concentration. This was not observed with casein coated wells in the absence of ecdysone suggesting that the Ag(s) may bind to the steroid. This could also have been the effect of high protein content although when a comparable concentration of casein was pre-incubated with G6 and 0.5 gland equivalents the absorbance values were not significantly different from G6 and 0.5 gland equivalents in the absence of added protein. Although this does not control for the spectrum of proteins and glycoproteins present in the PG homogenate it does indicate that the observed effect was probably not the result of non specific binding.

Partial Purification of G6 Immunoreactivity on a Sephadex G-50 Column

To isolate the component(s) of the PG which was immunoreactive and to get an approximate indication of mwt, PG tissue was homogenised and loaded onto a Sephadex G-50 column and eluted with PBS. Fractions were collected and used to coat ELISA plate wells and

assayed using the G6 Ab. The method was as follows.

Method

Ten pairs of W + 2 PG were homogenised in 100 ul PBS (pH 7.2, 0.15m) and spun in a microcentrifuge (Eppendorf 5412) for 5 mins. The supernatant was removed and loaded onto a Sephadex G-50 column (14 cm x 1 cm) which had previously been equilibrated with PBS. The column was run under gravity at 6.5 ml per hour. 600 ul fractions were collected. The fractions were assayed in triplicate, 100 ul of each fraction being used to coat each well of a rigid pvc ELISA plate (Titertek) and incubated overnight at $4^{o}C$. The ELISA protocol was as follows.

After coating overnight the fractions were discarded and 100 ul of 1% casein in PBS was added and incubated for 30 mins at $37^{o}C$. The blocking solution was discarded and the plate washed in 3 x 5 min changes of PBS/Tween 20 (0.05%). G6 ascites was diluted at 1:200 with PBS and was incubated for 1 hour at $37^{o}C$. The MAb was then discarded and the washing step repeated. 100 ul of the second Ab solution, 1:1000 goat anti mouse alkaline phosphatase (Sigma) in PBS/Tween 20, was added to each well and the plate was incubated for 1 hour at $37^{o}C$. The solution was discarded and the washing step repeated. The phosphatase substrate 'Sigma 104' (Sigma) was dissolved in 10% diethanolamine buffer (pH 9.6) at a concentration of 1 mg per ml and 100 ul of the resulting solution was placed in each well. The plate was allowed to develop at room temperature for 45 mins and the absorbance value for each well measured in a Titertek ELISA plate reader using a 405 nm filter.

Results

The histogram of immunoreactivity recovered from the Sephadex G-50 column can be seen in Fig 5.2. The immunoreactive peak occurred in the high mwt region, distinct from the region where ^{3}H-ecdysone eluted, and gave approximately fivefold higher absorbance values than ecdysone coated wells (2 ug per ml). Thus, the principal Ag in the PG recognised by G6 is distinct from ecdysone and is of high mwt. If ecdysone was present in the PG extract it was not detected. The upper limit of Sephadex G-50's molecular size fractionation range is around 30KD. The G6 Ag(s) was excluded from the gel and therefore has a mass greater than this figure. A second gel filtration system, Sephacryl S-300 superfine which discriminates molecules of up to 1.5 x 10^{3} KD, was used to estimate the mwt of the G6 Ag(s).

Partial Purification of G6 Immunoreactivity on a Sephacryl S-300 Column

The following experiments aimed at obtaining a mwt estimation for the G6 Ag(s) but also to gain insight into the tissue distribution and developmental profile during the fifth instar of Manduca.

A 14 x 1cm column was packed with Sephacryl S-300 superfine (Pharmacia) which had been pre-swollen and equilibrated with PBS (pH 7.2, 0.15m). Approximately 250 ug of protein (as determined by 260/280 nm analysis: see chapter III) was loaded onto the column for each developmental stage and tissue and used to examine the distribution of the G6 Ag(s). The amount of protein was standardised to allow for the temporal differences in PG protein content during the fifth instar (Stott, 1983) as well as variability

of tissues from different individuals. The column was run under gravity (6.5 ml per hour flow rate) and the ELISA assay of fractions standardised by the inclusion of a 2 ug per ml 20-HE-BSA coated well and the absorbance of that well was allowed to reach 0.3 before the ELISA plate was read. This reduced inter-assay variability. Haemolymph was bled from the abdominal "horn" and was combined from two individuals. The haemolymph was spun in a microcentrifuge (Eppendorf 5412) to remove haemocytes and was chilled on ice and loaded onto the column immediately. A few crystals of phenylthiourea (PTU) were added to each sample to inhibit tyrosinase activity. The data from the column runs is summarised in Figs 5.3 and 5.4.

Results

Fig 5.3 depicts the profiles of immunoreactivity recovered when ANC, FB and PG were subjected to S-300 gel filtration. No detectable immunoreactivity was recovered when ANC from W + 2 animals was separated. Two distinct peaks account for the immunoreactivity recovered from FB separations. Firstly there was a large mwt immunoreactive peak that eluted immediately after dextran blue and had an apparent mwt of 200-300 KD. The second peak recovered co-eluted with ecdysone and vitamin B-12 and was less than 10KD.

Gel filtration of PG extracts resolved a single peak of immunoreactivity which was eluted from the gel in the same position as the large mwt species recovered from the FB. The temporal profile of the PG peak shows that although immunoreactivity is detectable in feeding fifth instar larvae and in post-wandering animals there appeared to be maximal levels in PG taken from W + 2

larvae (Fig 5.3).

Analysis of haemolymph (Fig 5.4) detected two immunoreactive peaks that corresponded in position to those found in FB. There appeared to be little change in haemolymph titer of the 200-300KD Ag through the fifth instar but the relative levels of the smaller mwt peak increased. In feeding fifth instar larvae the small mwt peak was absent or undetectable but post wandering (W + 0) it was recovered and by day W + 3 the absorbance values attributable to the two immunoreactive peaks had reached parity (Fig 5.4). Exact quantitative comparison of the two Ags cannot be made from this data for a number of reasons including variability in column recovery and variability in the insects from which the samples were derived. Most importantly, direct quantitative comparison cannot be made between the quantities of high and low mwt peaks in the absence of information as to the relative affinity the G6 MAb exhibits for the two molecular species.

The high mwt molecule was investigated further by SDS-PAGE techniques.

SDS-Gradient-PAGE Methods

An SDS-gradient-PAGE gel was run with a gradient of between 5-12% acrylamide, according to standard protocols. (Laemmli, 1970)

Sample Preparation

Freshly dissected tissues were homogenised in TRIS/HCl (0.5M, pH 6.8) to give a final volume of 30 ul. The solution was spun (Eppendorf 5412) and the supernatant removed and mixed with an equal volume of sample buffer/bromophenol blue. The composition of the sample buffer was as follows; TRIS-HCl containing 10% SDS, 10 ul

2-ME per 40 ul of solution, and 20 ul of glycerol was mixed with bromophenol blue in TRIS/HCl to give 0.005% w/v. The samples were then either incubated at 37°C for 30 mins prior to loading or boiled for 2 mins and then loaded. Also some samples were acetone precipitated to establish whether or not this would provide a useful way of concentrating samples. Acetone was chilled on ice and five volumes of the acetone were added to one volume of the sample, vortexed and incubated for 30 mins at 4°C. The samples were spun in a microcentrifuge (Eppendorf 5412) and the supernatant discarded and the pellet resuspended in 30 ul TRIS/HCl. The samples were loaded onto the stacking gel along with standards (Sigma) which were prepared in the same loading solution as the samples. The gel was run for 4 hours at 300v.

Electroblotting

The electroblot tank was filled with transfer buffer (25 mM TRIS, 192 mM glycine plus 1.2 litres methanol to give a final volume of 4.8 litres). Filter paper was soaked in the buffer. The gel was carefully placed on half the filter paper covering the cathode and then nitrocellulose (pore size 0.4 um) cut to the size of the gel and soaked in buffer was placed over the gel. All air bubbles were expelled and the remaining half of the filter paper folded over to cover the nitrocellulose. The transfer cassette was then filled out with scouring pads, secured and placed in the tank and run overnight at constant current of 0.3A (approximately 80v). A cooling coil was used to prevent overheating. Once transfer was complete the gel and nitrocellulose were removed. Standard tracks on the nitrocellulose were cut and stained in a 0.5% solution of amido black (w/v) prepared in 5% acetic acid (v/v), 50% methanol (v/v) for 5 mins.

The nitrocellulose was then washed for 1-2 mins in water and destained in 5% (v/v) acetic acid 50% (v/v) methanol until the background was clear and the standard bands appeared blue.

Staining of Nitrocellulose Sample Tracks with G6 MAb

The nitrocellulose was washed briefly in PBS + 1% casein then blocked in fresh solution for 90 mins at 37°C and shaken gently throughout. The G6 MAb was diluted in PBS + 1% casein to give a dilution of 1:100. The nitrocellulose was incubated with the Ab for 3 hours at 37°C whilst shaking. The paper was then washed in 5 x 5 min changes of PBS + 1% casein. The second Ab enzyme conjugate (goat anti-mouse peroxidase - Sigma) was diluted 1:1000 in PBS + 1% casein and incubated and shaken at 37°C for 2 hours. The washing step was repeated. The substrate (50 mg DAB + 30 ul H_2O_2 in 100 ml of PBS (w/v/v)) was added for 15 mins at room temperature whilst shaking and the substrate solution replaced and incubated for a further 15 mins. The nitrocellulose was then washed in several changes of water and dried at room temperature.

Results

Fig 5.5 represents five different samples and sample treatments. Lane 1 contained haemolymph (W + 2) which had been acetone precipitated. No bands are visible. Lane 2 contained haemolymph from W + 2 animals which had been incubated at 37°C with the dye and not boiled or acetone precipitated. Under these conditions a band is clearly visible which corresponds to a mwt of approximately 200 KD. Lane 3 contained boiled haemolymph (W + 2). No bands are detectable. Lane 4 was boiled acetone precipitated PG (10 pairs, W + 2). Again no bands can be seen. Lane 5 represented

ANC which had not been boiled. No bands are visible.

Boiling and acetone precipitation both rendered the Ag 'unrecognisable' by the MAb. The only successful treatment was incubation at 37°C prior to loading. This therefore precluded sample concentration by the acetone method. In subsequent gels, although bands were visualised they were close to the detection limit of the system and so did not readily photograph. However the results from freshly stained gels are summarised in Table 5.2 and give both the banding patterns observed and mwt estimations.

PG samples all gave a 200-300 KD band which occurred in W + 1, W + 2 and W + 3 stage animals using ten pairs of glands in each lane. This mwt estimation is in keeping with the estimate derived from the Sephacryl S-300 chromatography. A second, fainter, band was detected with a mwt of 32 KD. Similarly FB (W + 2) gave the same bands of 42 and 52 KD respectively. Haemolymph gave the same banding patterns as FB but no 52 KD band was visualised. W + 3 and W + 4 haemolymph gave the strongest bands when equal volumes (30 ul) were run by comparison with days W + 0, W + 1 and W + 2. ANC (W + 2) did not appear to contain detectable levels of H6 Ag(s).

In conclusion the high mwt G6 Ag has a mwt of 200-300 KD as determined by both gel electrophoresis under denaturing conditions and Sephacryl S-300 gel filtration. Additional lower mwt bands of 32, 42 and 52 KD were also resolved by gel electrophoresis (SDS-PAGE) but not by gel filtration.

Some Biochemical Characteristics of the High and Low Mwt G6 Ags

The two peaks of immunoreactivity detected by Sephacryl S-300 chromatography were the 200-300 KD Ag which was present in PG, haemolymph and FB of W + 2 animals and the less than 10 KD Ag which

was only detected in FB and haemolymph. An attempt was made to investigate some of the biochemical characteristics of these Ags.

Method

For the large mwt Ag ten pairs of W + 2 PG were separated on the Sephacryl S-300 column and the fractions collected. A second column run was performed and the fractions collected and run in parallel with the first. For each treatment 2 x 100 ul samples were used from each fraction and the treatments were as follows; one set of fractions were left untreated. The second set were incubated, 37°C, 2h, with trypsin (4 units per 100 ul of fraction - Sigma) plus trypsin inhibitor (0.005 mg per 100 ul of fraction - Sigma). The third set of fractions received 4 units of trypsin per 100 ul of fractions, also 37°C, 2h, and the final set were boiled for 2 mins. Following treatment the fractions were placed in the wells of a rigid pvc ELISA plate (Titertek) and incubated overnight at $4^{o}C$. The ELISA plate was then developed in the standard manner described above, and read at 405nm. The results are summarised in Fig 5.6. All the data is from a single ELISA with all the fractions run in parallel. Each histogram bar represents the mean of two determinations. All the determinations of replicates within a fraction fell within $\pm$ 5% of the mean.

Low mwt G6 Ag was also investigated. 100 ul of haemolymph from W + 3 animals was separated on the Sephacryl S-300 column and treated in an identical manner to the PG fractions. Again two chromatography runs were performed and the same number of replicates analysed. Table 5.3 summarised the results of the absorbance values for the peak of the low mwt G6 activity.

Results

The high mwt G6 Ag will be discussed first. Untreated fractions gave the typical profile of G6 immunoreactivity with the major peak occurring in fraction 6. The same profile was seen with trypsin plus trypsin inhibitor treatment. When trypsin alone was incubated with the fractions the peak of G6 immunoreactivity was totally absent. Similarly, boiling of the fractions for 2 mins rendered the G6 Ag 'unrecognisable' by the G6 MAb.

The low mwt G6 Ag detected by the G6 MAb was unaffected by trypsin/trypsin inhibitor treatment and also trypsin treatment alone, as determined by immunoreactivity. However boiling of the samples rendered the low mwt Ag 'unrecognisable' to the MAb and the absorbance values were not significantly different from background.

In vitro Assay System

It was of interest to explore the possibility that the immunoreactive G6 molecules exert an influence on secretion by the PG in short term culture. Ecdysone secretion into the culture medium was measured by RIA.

Method

PG pairs were dissected from W + 1 animals and placed in sterile Grace's culture medium (Flow) to wash at room temperature while the remaining glands were dissected. (Glands were never washed for longer than 15 mins prior to being placed under the final culture conditions). In a given experiment one gland was used as control and the contralateral gland as the experimental. This controlled for many variables that are associated with the comparison of glands from different individual animals (Stott, 1983). Glands were

cultured individually in a volume of 25 ul in the wells of micro-titertek 60 well plates (Flow-Titertek) and the plates were humidified and incubated for 2 hours at 26°C. At the end of the incubation period the glands were removed and the culture medium extracted in methanol.

Preparation of Culture Medium for RIA

125 ul of methanol (4°C) was added to 25 ul of culture medium from each well, vortexed and spun in a microcentrifuge (Eppendorf 5412) for 5 mins. The methanolic supernatant was dried down under nitrogen and stored at -20°C.

RIA Protocol

To each dried down aliquot of extracted culture medium 100 ul BB + 1% BSA was added containing approximately 4000 cpm ^{3}H-ecdysone. The tube was vortexed and to this was added 100 ul of DUL-2 Ab (see chapter III) in BB + 1% BSA to give a final dilution of 1:200, which was the dilution determined from a binding analysis that would bind 50% cpm. The tubes were vortexed and incubated overnight at 4°C. Precipitation of the Ab was performed, by the addition of 200 ul SAS (4°C) to each tube followed by immediate vortexing. The tubes were incubated for 30 mins at 4°C then spun at 4500 rpm (Beckman, J6) for 15 mins at 4°C. The supernatants were carefully discarded and the pellet resuspended in 40 ul 50% SAS in BB (v/v), vortexed, incubated at 4°C for 30 mins then spun as described above. The supernatant was discarded and the pellet resuspended in 200 ul distilled water and transferred to polyethylene scintillation vials (Packard) along with 5 ml liquid scintillant. The samples were counted in a liquid scintillation counter (LKB, Rackbeta). A

standard curve was constructed for each assay in the range of 0.1 ng - 10 ng ecdysone using the method outlined above.

Basal Secretion Rates of PGs Under in vitro Culture Conditions

The aim of this experiment was to obtain an estimation of basal ecdysone secretion rates by the PG and to see if it was possible to stimulate elevated levels of secretion in the presence of the FB factor described by Greutzmacher et al (1984). The nature of this factor is not yet known. In the present experiments, following Greutzmacher et al (1984) we used Grace's medium preconditioned by culturing FB from day W + 3 caterpillars. It was unlikely that secretion rates would be optimal under the conditions employed here (incubation volume etc were not optimised), but provided that the PG responded to the FB factor with an increased rate of ecdysone secretion, this would prove adequate for the investigation of any effects of the Ags recognised by the G6 MAb on ecdysone secretion. The results are summarised in Table 5.4.

Results

As predicted, the FB conditioned medium significantly increased ecdysone secretion rates by the PG.

In vitro Ecdysone Secretion by the PG in the Presence of the G6 Ags

Method

200 ul of W + 3 haemolymph was separated on the Sephacryl S-300 column (described previously) and 100 ul of each fraction was assayed in the G6 ELISA. The fractions which coincided with the peaks of the two immunoreactive molecules were retained for

incorporation into the in vitro PG culture system (see above). The culture conditions were as follows; one gland plus 15 ul Grace's culture medium plus 10 ul PBS. The contralateral gland plus 15 ul Grace's medium plus 10 ul fraction (PBS). The culture conditions and analysis were as described above.

Results

The results are summarised in Table 5.5. The presence of the low and high mwt G6 immunoreactive peak fractions did not significantly affect ecdysone secretion rates by the glands.

Effect of the G6 MAb in vivo

The experiment described in this section addresses the question of whether or not injection of the G6 MAb in vivo into developing fifth instar larvae has any detectable effect on the pattern of normal development.

In vivo Injection Protocol

Animals selected on day W + 0 were matched in pairs on the basis that they had wandered within a 3 hour period of each other. The experimental half of the pair was injected with 25 ul of G6 ascitic fluid (0.65 mg Ig) with the other half of the pair receiving a comparable concentration of rabbit anti-mouse Ig in PBS (Sigma). (This control is not strictly comparable with the experimental animals because it is not a mouse MAb of the IgM class. However there is difficulty in selecting a mouse MAb that would have been guaranteed not to have an effect in vivo. However the control was considered to be more comparable than simply injecting control

animals with PBS). The animals were injected using a 25 G needle (Hamilton syringe) dorsally above the level of the spiracles between the 5th and 6th abdominal segments. Daily injections were given at the same time of day as the initial injection and the animals were examined daily for the onset of dorsal bar formation and tanning of the first abdominal segment. These events occur 24 hours and 1 - 2 hours prior to pupal ecdysis (Truman et al, 1980). In this manner the temporal course of development from post-wandering to pupation was monitored. Once pupation had taken place injections were no longer given.

Results

The results are summarised in Fig 5.7. Two animals pupated 24 hours after their controls when injected with the G6 MAb. Two animals pupated approximately 12 hours after their controls and one animal pupated at the same time as its control. A group of animals which were untreated but which had wandered within 3 hours of each other (n = 5 pairs) were monitored to see what range of pupation times were normally exhibited. From the 10 animals observed the maximum time between pupation of matched pairs was 5.5 hours. The mean was 2.75 hours. It therefore seems that the G6 MAb injection results were not attributable to normal variation and that delays in development of 12 and 24 hours are significant. The fact that one pair of animals did not differ in their pupation times probably indicates variability in the way the G6 MAb is degraded by different animals or is due to differences in, eg, blood volume, etc.

Effect of Neck Ligation on the G6 Ags

It was of interest to observe the effect on the immunoreactivity profiles of haemolymph from caterpillars which had been neck ligated on day W + 0, thus removing the influence of the brain/CC/CA derived factors on the haemolymph. Additionally, the effect of the G6 Ags was investigated by neck ligation on day W + 0, coupled with JH treatment to investigate whether or not JH is involved in the G6 Ags presence in the haemolymph.

Protocol

Day W + 0 *Manduca* caterpillars were neck ligated using cotton thread. Half the animals were treated topically with 10 ul acetone along the mid-dorsal region using a microcapillary tube. The other half of the animals were neck ligated in the same manner but topically treated with 100 ug of the JH analogue ZR515 in 10 ul acetone. On day W + 2 the haemolymph from the animals was collected and spun in a microcentrifuge (Eppendorf 5412). 200 ul of the cell free haemolymph was mixed with a few crystals of PTU and loaded onto the S-300 Sephacryl column (described elsewhere). The fractions were analysed in the ELISA system using the G6 Ab to detect immunoreactivity. The results are summarised in Fig 5.8.

Results

The results show the immunoreactive profile for three treatments; non-ligated, untreated animals, neck ligated acetone treated animals and neck ligated ZR515 treated animals. Haemolymph samples from n = 4 W + 2 animals for each treatment were pooled and run on the Sephacryl S-300 column and the fractions assayed in a single ELISA.

The untreated animals haemolymph exhibited the typical W + 2 haemolymph immunoreactivity profile, that is two discreet peaks of activity of 200-300 KD and less than 10 KD (see also Fig 5.4). When the animals were neck ligated on day W + 0 and topically treated with acetone the profile of immunoreactivity of haemolymph assayed on day W + 2 corresponds with that derived from untreated, non-ligated, control animals. The magnitude of immunoreactivity, as expressed in absorbance units, was larger than that seen with control animals but this is attributable to variation in immunoreactive material seen in haemolymph and tissue samples from different individuals. What is important is the number and mwt of the two peaks. These correspond exactly with the G6 200-300 KD and less than 10 KD Ags. Therefore neck ligation does not reduce or prevent the production of the two Ags. When day W + 2 animals were neck ligated and topically treated with ZR515 the immunoreactive peak associated with the high mwt G6 Ag was absent. The low mwt G6 Ag peak was less clearly defined than in untreated or acetone treated controls. There was some immunoreactivity within the predicted fractions but it was not discreet.

Staining of PG Cells and Sections with G6 MAb

PG sections were stained with G6 MAb according to the protocol described in detail in chapter IV. Also whole PG cells were stained with the G6 MAb according to the following protocol.

Staining Protocol for Whole Cells

PG (W + 2) were carefully dissected out and washed in Grace's culture medium (3 x 5 min changes). The glands were then stained (without fixation) with the G6 MAb (1:100 in PBS) according to the

methodology described for PG sections in chapter IV. The cells were blocked with normal rabbit serum and a rabbit anti-mouse Ig-FITC conjugate was used to visualise the cells (Sigma).

Results

The PG sections (Fig 5.9) exhibit weak, diffuse, heterogeneous staining mainly in the cytoplasm and cell periphery. From the staining of whole cells distinct patches of bright stain on the cell surface can be seen (Fig 5.10).

Discussion

This chapter reports the isolation of a MAb which was raised against an immunogen of crude PGs. The MAb (G6) was detected by its ability to bind ecdysone in an ELISA and was subsequently used to detect and characterise the native Ag in the PG. The Ag isolated from the PG was also found to occur in FB and haemolymph. Interestingly, there was also an additional Ag recognised by the Ab in FB and haemolymph which was absent from PG extracts. The approximate mwts of the two Ags were 200-300 KD and less than 10 KD respectively.

The Nature of the PG Ag

The MAbs from the fusion were screened against free ecdysone in the ELISA. However it is unlikely that free ecdysteroid in the PG was the Ag which elicited the MAb (G6) because ecdysone has a mwt of approximately 500D and is therefore not inately antigenic. Also from the work of King et al (1974) it appears that the PG do not store the steroid but secrete it as it is synthesised. They were

unable to detect free ecdysteroid in the PG by RIA analysis. Therefore the Ag within the PG, against which the G6 MAb is directed, must either share a common antigenic determinant with ecdysone or be some type of protein-steroid complex that is a naturally occurring hapten-carrier association. An important question is whether the G6 MAb is detecting an Ag which is functionally important within the PG or involved in some aspect of steroid secretion, or whether it is simply a non-homologous cross reaction of no relevance to PG function? In an attempt to gain more understanding of the G6 Ag some characterisation of the molecule(s) was undertaken.

Evidence for the Nature of the G6 PG Ag

The mwt of the Ag was estimated by two independent techniques, gel filtration chromatography and electrophoretic separation under denaturing conditions coupled with immunoblotting. Both estimates were in agreement that the PG Ag recognised by the G6 MAb was in the order of 200 - 300 KD. Some biochemical properties of the Ag were then investigated by trypsin treating the partially purified molecule and by boiling it and observing whether or not it was still recognised by the G6 MAb after such treatment. Following trypsin digestion the G6 MAb did not bind to the Ag. This suggests that the molecule is probably a protein and implies that the antigenic site of the molecule is susceptible to trypsin cleavage. If the molecule is a protein that resembles ecdysone by virtue of cross reactivity at an antigenic determinant this would be in keeping with this result. However, if the Ag was a steroid-protein complex it could still be rendered unrecognisable to the MAb if the MAb is

recognising the steroid in its conformational state when bound to the protein.

The antigenic site recognised by the MAb is unstable to heating to 100°C and also to acetone precipitation.

Tissue Distribution of the G6 Ags

Despite the fact that PG tissue had been the immunogen against which MAb G6 was directed, the Ab recognised the PG Ag in two other tissues, namely FB and haemolymph. However, it was not ubiquitous to all insect tissues and the Ag failed to be detected in a homogenate of ANC.

In addition to the 200-300 KD G6 Ag there was also a lower mwt species detected by the MAb in FB and haemolymph. By gel filtration chromatography this molecule had an apparent mwt of less than 10 KD. It remained a possibility that the low mwt molecule with which G6 cross reacted could be ecdysteroid or an ecdysteroid metabolite or conjugate. When its biochemical nature was investigated it was still bound by the MAb when trypsin treated but was not recognised if it was boiled. This was in contrast to free ecdysone which was found still to be recognised by the G6 MAb when boiled. Also the low mwt Ag had the capacity to bind to rigid pvc ELISA plates to which free ecdysone in ug quantities failed to bind. It is therefore unlikely that the <10 KD Ag is free ecdysone.

When separated under denaturing conditions by SDS-gradient-PAGE all three tissues in which the large mwt G6 Ag occurred showed 32 KD and 42 KD bands of immunoreactivity. In addition a 52 KD band occurred in FB. These bands were all on the detection limit of the system and were not as distinct as the high mwt band. Either these represented molecules within these tissues that on denaturation

became recognised by the G6 MAb, as they were not detected in the native gel filtration/ELISA system, or more likely that they represented sub units of the 200-300 KD protein. Perhaps somewhere in the region of 6-8 of these sub units comprise the final molecule.

The Possible Functional Roles of the High Mwt and Low Mwt Ags

An indication of the role of a molecule in developmental and physiological processes is its capacity to be regulated. The developmental profile of the Ags was therefore examined in PG and haemolymph during the fifth instar. The results have to be viewed with some caution because there is difficulty in standardising all the variables in the chromatography and subsequent ELISA. These include the inherent variation in quantities of the Ag in individual animals and variation in recovery from separate column runs. Perhaps the only way to reduce these variables' influence on the absorbance profiles would be to increase the sample size and to establish some system to estimate recovery. This would then allow an accurate measurement of recovery from the column and enable an assessment to be made of whether or not this error is systematic. However, within these limitations, the assay was standardised for both quantity of protein loaded on column and also for intra-assay variation in the ELISA, so some tentative conclusions can be made.

The large mwt Ag occurs in PG extracts during the feeding stage of the fifth instar but reaches maximal levels on day W + 2. This coincides with the rise in ecdysteroid titer just prior to pupation. A similar trend is seen in haemolymph samples analysed during the fifth instar.

The low mwt Ag, however, exhibits a much more dramatic regulation during the final larval instar of Manduca. It occurred

in undetectable quantities on day 3 of the fifth instar and gradually increased throughout the post-wandering period with maximal levels being achieved by day W + 2 and W + 3. Certainly the low mwt molecule can be accurately compared on the basis of its relative absorbance values by comparison to the absorbance values of the high mwt molecule on a given day. It can be seen to increase in relation to the high mwt Ag to reach parity on days W + 2 and W + 3. Most dramatic of all though is the FB from day W + 2 which has 2 x absorbance value attributable to the low mwt Ag by comparison with the high mwt molecule. A proposed area of future study would be the developmental profile of the two mwt Ags in FB. Since days W + 2 and W + 3 are those on which the pre-pupal ecdysteroid titer reaches its peak, this data is not incompatible with the low mwt Ag being a steroid conjugate or metabolite.

Several authors, eg, Greutzmacher et al (1984); Stott (1983), have used in vitro PG incubation systems to study the influence the PG environment has on secretion rates of ecdysone. It was therefore considered a useful system in which to introduce the two mwt form of the G6 Ag and to measure ecdysone levels secreted by the glands. Any differences in secretion rate in response to the two factors could provide insight into the two molecules putative functional roles. However, when these experiments were performed using partially purified gel filtration fractions as a source of the two factors no significant effect was detected in ecdysone secretion rates by the glands. This suggests that either the molecules do not influence PG ecdysone secretion or that the amounts of the two G6 Ags that were included were too low to have a significant effect. If their incapacity to influence PG secretion is genuine this would preclude the possibility that the factor(s) act as sterol precursors

or transporting proteins to the glands (Greutzmacher et al, 1984).

When G6 MAb was injected into W + 0 Manduca larvae in vivo there was evidence of a delay in the onset of pupation in the majority of animals injected. However, there are at least two possible phenomena which could explain this result. Firstly, this could reflect that the G6 Ag(s) are required for normal development or alternatively this is merely a reflection of the capacity of the MAb to bind free ecdysteroid, hence delaying pupation.

In vivo injection of MAb is always subject to a multitude of variables, the majority of which cannot be controlled for. Certainly in work using an anti-progesterone MAb to block pregnancy in mice (Wright et al, 1982) the timing of MAb injection, the quantity and the rate of injection all influenced whether or not the MAb was effective. Similarly, in Manduca in vivo injections these factors will also be important and dose of Ig injected could be of significance as the rate of breakdown of the MAb by the caterpillar is an unknown parameter.

Further evidence that the G6 Ag(s) are not sterol precursor transporting proteins analogous to the FB stimulatory factor (Greutzmacher et al, 1984; see also chapter I of this thesis) are provided by neck ligation experiments performed on day W + 0 animals. The presence of immunoreactive molecules (both high and low mwt forms) persisted even when neck ligation was performed on day W + 0 and blood analysed on day W + 2. According to Greutzmacher et al (1984) in the absence of the CC/CA the FB factor is absent from the W + 2 animal. This is in direct contrast to the situation with the G6 Ags. Furthermore, and perhaps of greatest interest, when JH was applied, in the form of the analogue ZR515, to

ligated animals the two mwt forms were absent in the blood on day W + 2. This was most pronounced for the high mwt form but also true, to a less well defined extent, for the low mwt Ag. Because JH indirectly promotes ecdysone secretion by the PG via the mediation of the FB factor (Greutzmacher et al, 1984) the result obtained here strongly suggests that the two Ags are not involved in the process of ecdysone secretion.

There remains the possibility that the high mwt Ag is an ecdysteroid binding protein which perhaps protects the steroid from degradation. There is evidence of such proteins in Drosophila hydei (Butterworth and Berendes, 1974) in which a 120 KD protein specific for ecdysone occurs. A high mwt 20-hydroxyecdysone specific protein occurs in Locusta migratoria (Feyereisen, 1977). The possibility that the G6 protein functions in a similar manner could be readily tested by incubation of ^{3}H-ecdysteroid with haemolymph and subsequently separating the blood by chromatography. The fractions could be analysed to establish whether or not counts are bound in the region of the immunoreactivity peak.

Irrespective of any possible role(s) in steroidogenesis the two G6 Ags are of considerable interest by virtue of their tissue distribution, temporal pattern through the fifth instar and their biochemical characteristics. Of particular importance is their apparent degradation and/or supressed production in JH treated animals. The MAb, G6, provides a unique tool for the purification of both molecular species and will be invaluable in their ultimate functional characterisation.

Table 5.1 Some Properties of the IE9 and G6 MAbs.

Cell Line	Ig Class	Variation in ELISA Replicates (n = 10) Absorbance ± SE	Ig Concentration in Ascitic Fluid mg per ml
IE9	IgM	0.89 ± 0.37	10.5
G6	IgM	0.95 ± 0.07	26.75

Table 5.2 Results of SDS-gradient-Page Analysis of *Manduca* Tissues During the Fifth Instar.

Tissue	Stage of Fifth Instar	Mwt of Band (KD)
Nerve cord	W + 2	No bands
Fat Body	W + 2	200 - 300 52 42 32
Prothoracic Glands	W + 1	200 - 300 32
	W + 2	200 - 300 32
	W + 3	200 - 300 32
Haemolymph	W + 2	200 - 300 42 32

Table 5.3 Some characteristics of the low mwt G6 Ag in the ELISA system. All absorbance values are for replicates.

Treatment	Peak Absorbance (405 nm) $\bar{x}$
Untreated	1.9
Trypsin/Trypsin Inhibitor	1.85
Trypsin	1.8
100°C (2 mins)	0.35
Background	0.30

Table 5.4 *In vitro* culture of PG with Grace's culture medium (control) and day W + 3 FB conditioned Grace's medium (experimental). The results are expressed as the mean ± SE. The experimental ecdysone secretion differs significantly from control ecdysone secretion in the Wilcoxon matched pair analysis at better than the 1% level.

Table 5.5 *In vitro* culture of PG with PBS + Grace's culture medium (control) and high and low mwt G6 Ag column fractions (experimental). The results are expressed as mean ± SE. The experimental and control secretion rates did not differ significantly.

IV	Ng Ecdysone/2 hours/gland	
	Control	Experimental
8	1.4 ± 0.12	3.8 ± 0.82

G6 Ag	IV	Ng Ecdysone/2 hours/gland	
		Control	Experimental
Low mwt	6	3.5 ± 0.56	2.28 ± 0.69
High mwt	6	3.9 ± 1.4	3.8 ± 1.4

Fig 5.1 Prothoracic Gland extract inhibition of binding of G6 MAb in the ELISA. The points on the graph represent the mean absorbance value for two replicates. The mean variation between replicates was $\pm$ 6.5%.

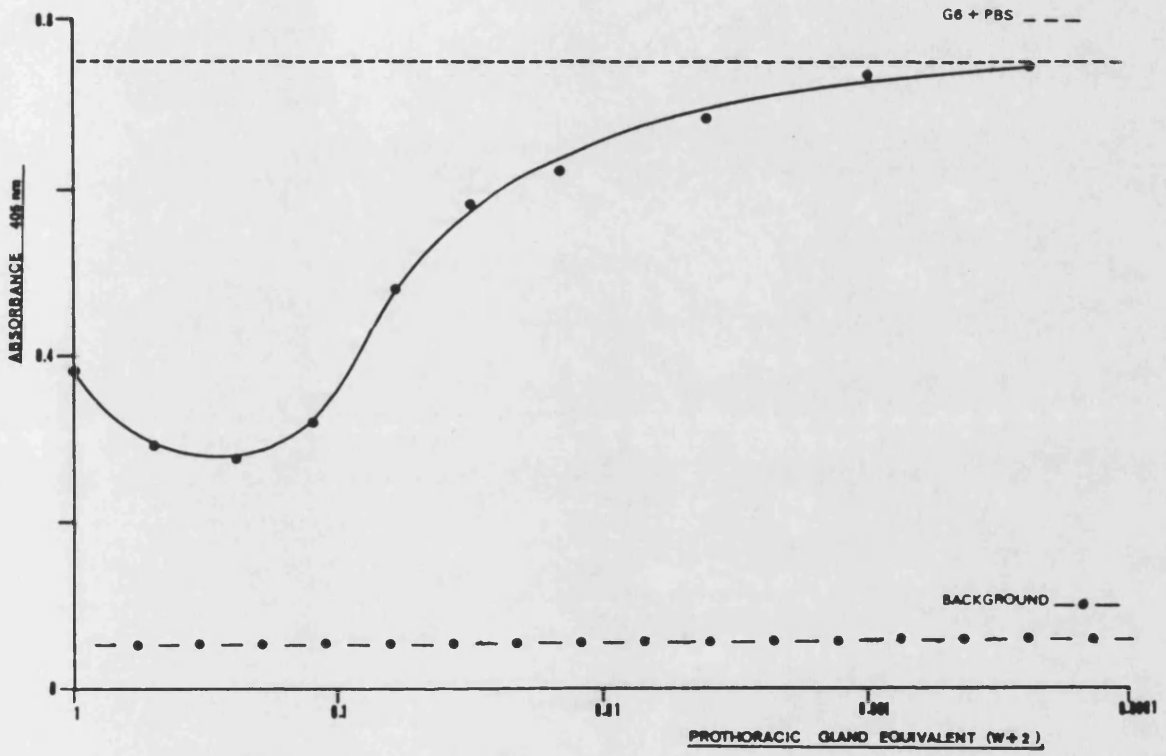

G6 + PBS
BACKGROUND
ABSORBANCE 405 nm
0.8
0.4
0
1
0.1
0.01
0.001
0.0001
PROTHORACIC GLAND EQUIVALENT (W+2)

Fig 5.2 Partial purification of G6 immunoreactivity on a Sephadex G-50 column. Each bar on the histogram represents the mean of triplicate determinations. All replicates fell within $\pm$ 7% of the mean.

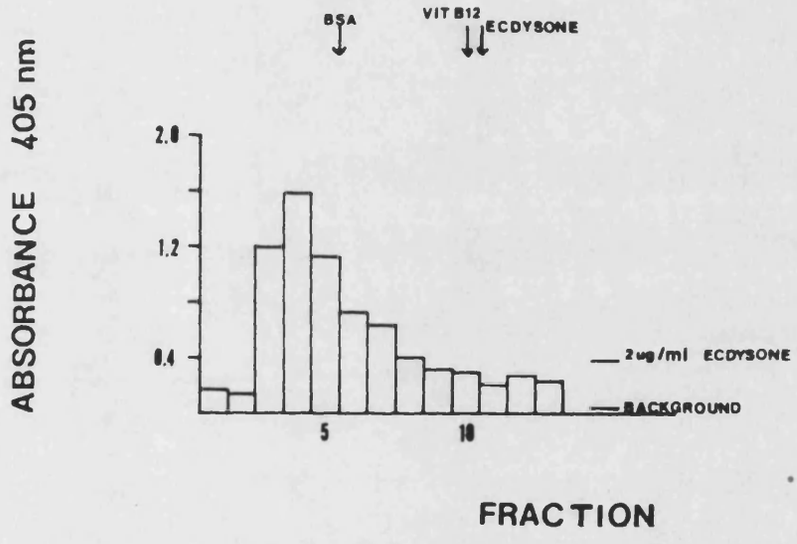

BSA
VIT B12
ECDYSONE
ABSORBANCE 405 nm
2.0
1.2
0.4
5
10
2 ug/ml ECDYSONE
BACKGROUND
FRACTION

Fig 5.3 Immunoreactivity profiles of tissue extracts separated on a Sephacryl S-300 superfine column. The histograms represent the profiles for ANC, FB and PG extracts throughout the fifth instar.

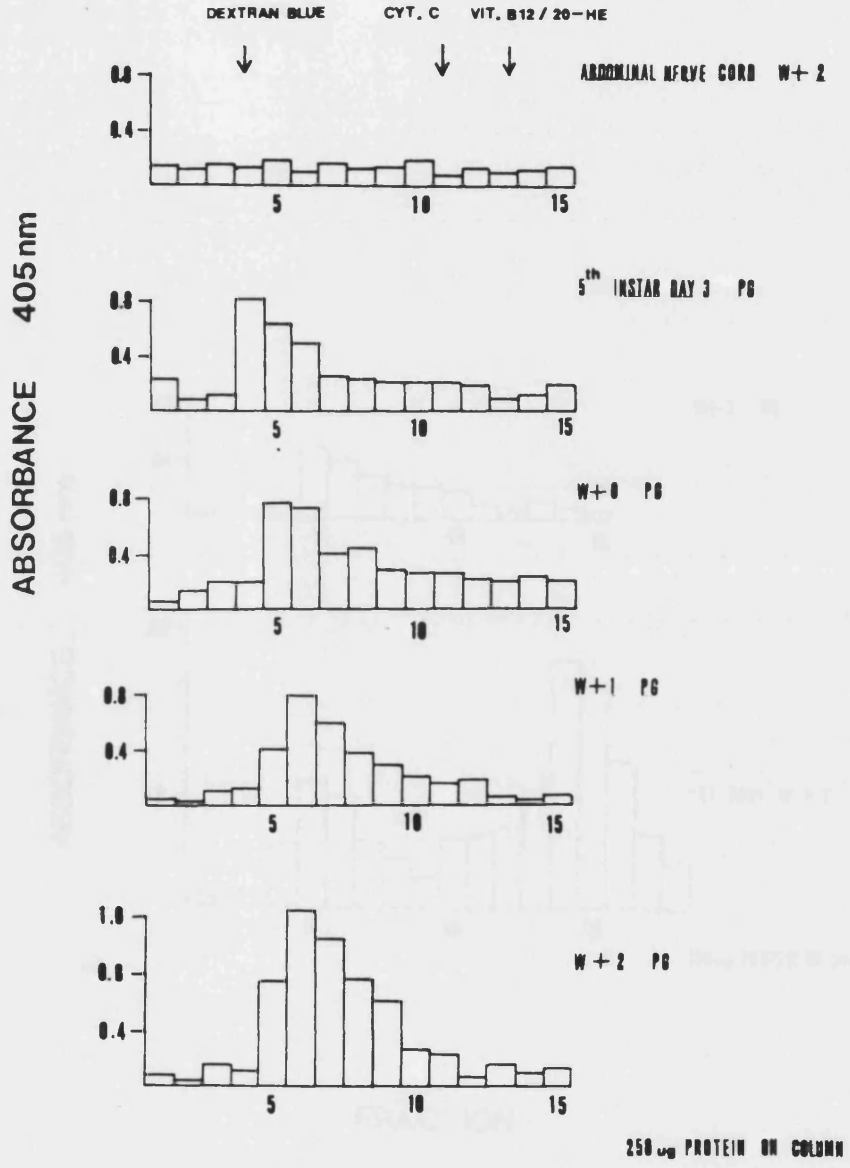

DEXTRAN BLUE
CYT. C
VIT. B12 / 20-HE
ABDOMINAL NERVE CORD W+ 2
5th INSTAR DAY 3 PG
W+0 PG
W+1 PG
W + 2 PG
0.8
0.4
1.0
0.6
5
10
15
250 µg PROTEIN ON COLUMN
ABSORBANCE 405nm
FRACTION

Fig 5.4 Immunoreactivity profiles of tissue extracts separated on a Sephacryl S-300. The histograms represent the profiles for haemolymph extracts throughout the fifth instar.

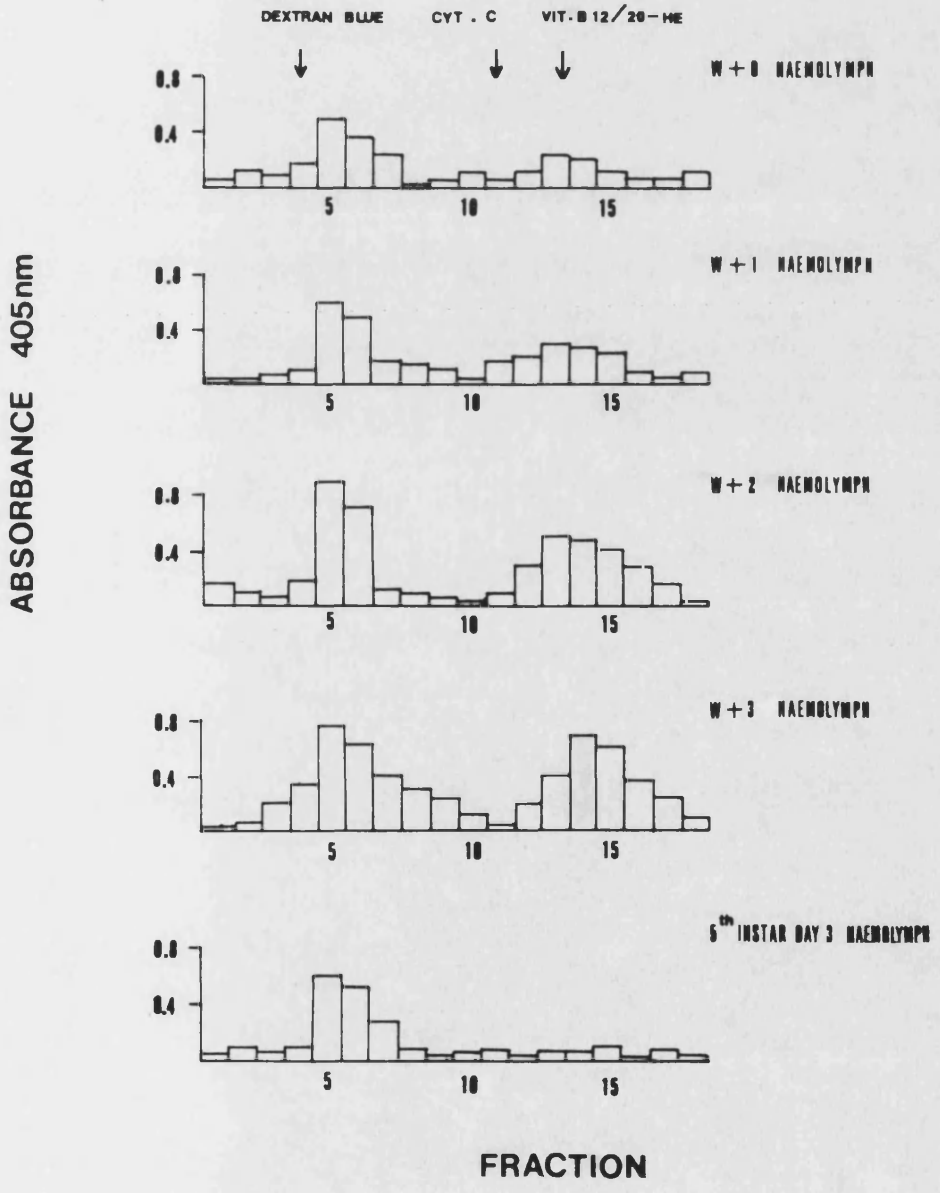

DEXTRAN BLUE
CYT . C
VIT-B 12/20-HE
W + 0 HAEMOLYMPH
W + 1 HAEMOLYMPH
W + 2 HAEMOLYMPH
W + 3 HAEMOLYMPH
5th INSTAR DAY 3 HAEMOLYMPH
0.8
0.4
5
10
15
ABSORBANCE 405nm
FRACTION

Fig 5.5 Photographic representation of nitrocellulose staining using the G6 MAb in an SDS-gradient-PAGE system. Lane 1 contained W + 2 haemolymph which had been acetone precipitated. Lane 2 contained haemolymph from W + 2 animals which had not been boiled prior to loading. Lane 3 contained boiled haemolymph from W + 2 caterpillars and Lane 4 contained acetone precipitated boiled haemolymph from day W + 2 caterpillars. Lane 5 contained ANC extract which had not been boiled.

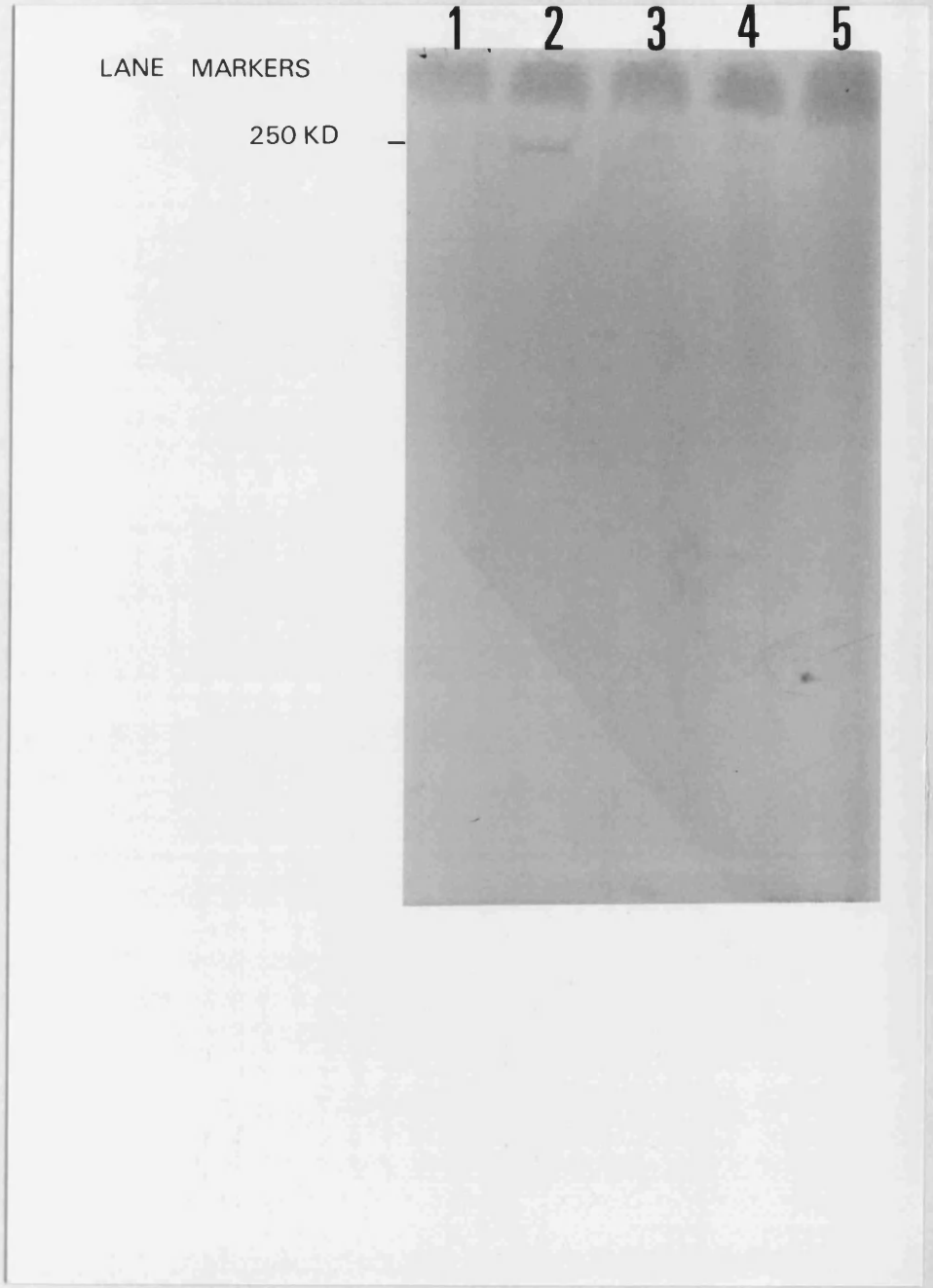

1
2
3
4
5
LANE MARKERS
250 KD

Fig 5.6 Partial biochemical characterisation of the 200 - 300 KD G6 Ag on the basis of immunoreactivity in the Sephacryl S-300 separation system in combination with ELISA.

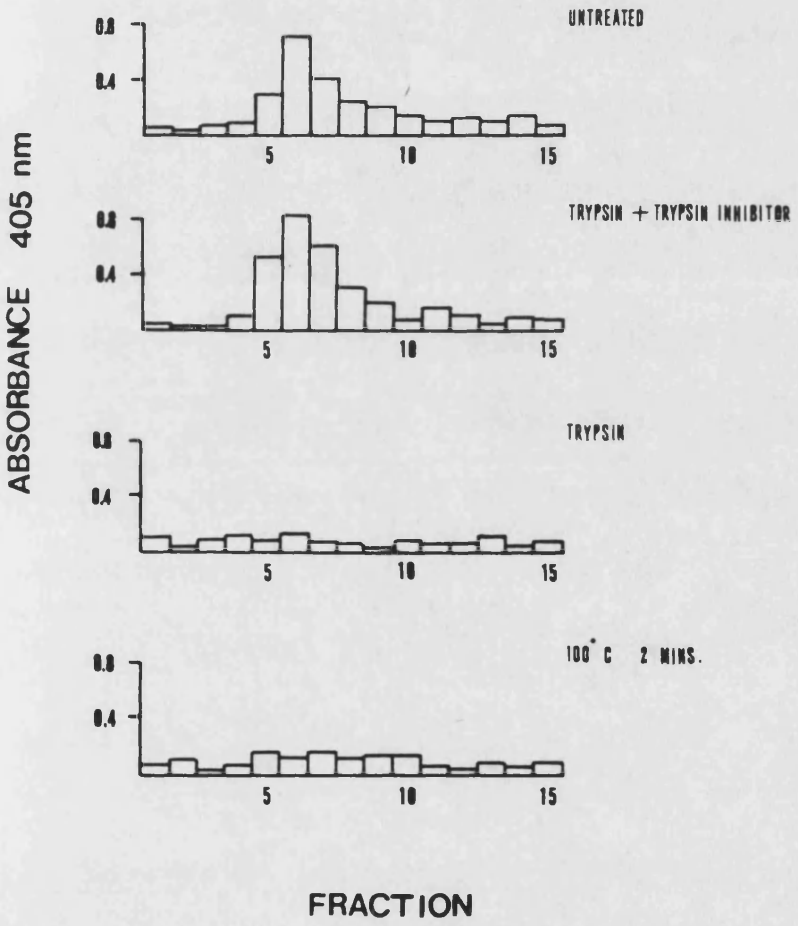
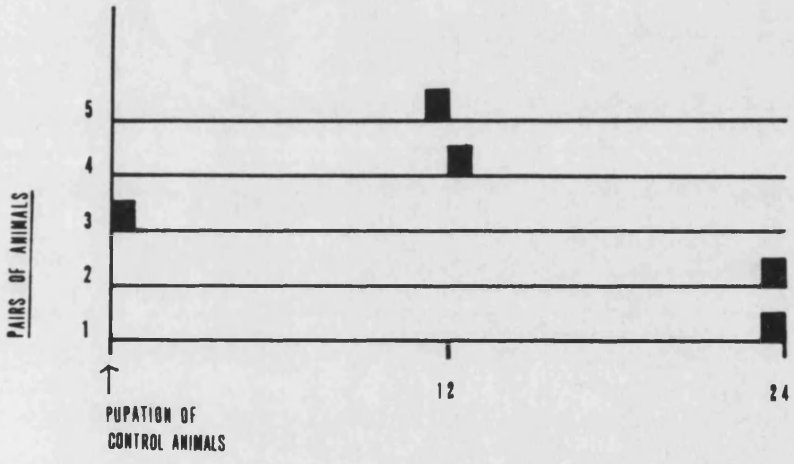

ABSORBANCE 405 nm
UNTREATED
TRYPSIN + TRYPSIN INHIBITOR
TRYPSIN
100° C 2 MINS.
0.8
0.4
5
10
15
FRACTION

Fig 5.7 Pupation times of animals injected with G6 MAb as compared to matched control animals. Animals had received daily injection of Ab up to the time of pupation.



TIME AFTER PUPATION OF CONTROL ANIMALS (HOURS)

Fig 5.8 Analysis of haemolymph samples derived from day W + 2 animals which had been neck ligated on day W + 0. The blood samples were analysed for immunoreactivity with the G6 MAb in the Sephacryl S-300/ELISA system. The histograms represent pooled haemolymph samples from 4 animals.

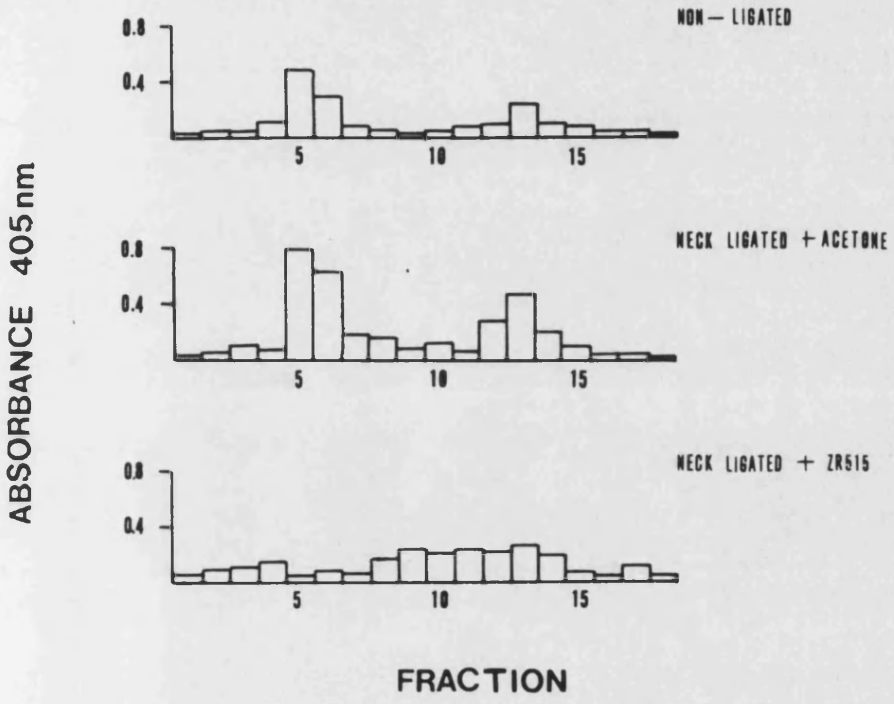
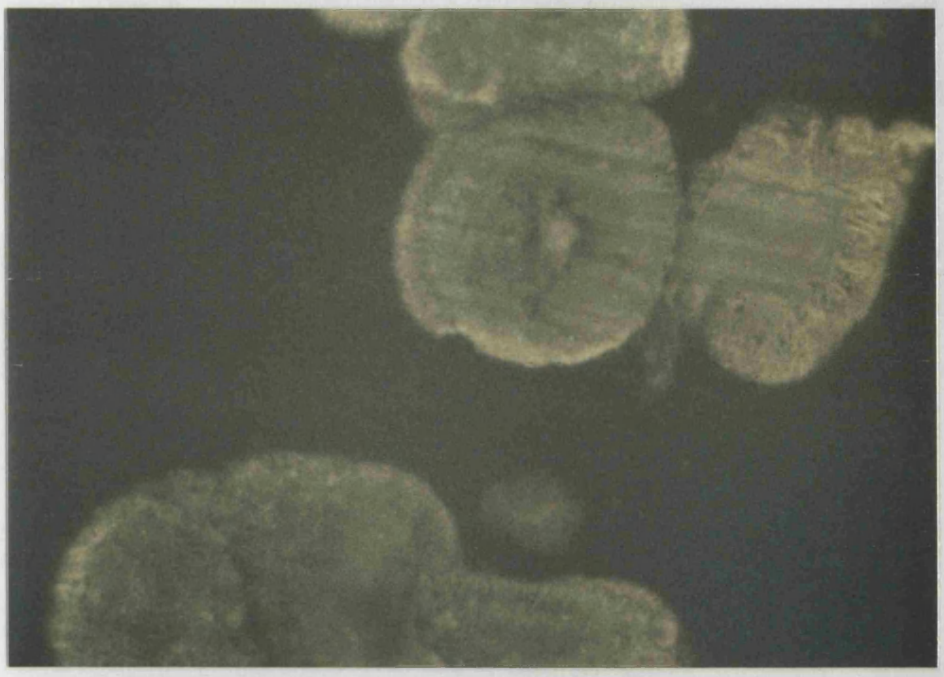
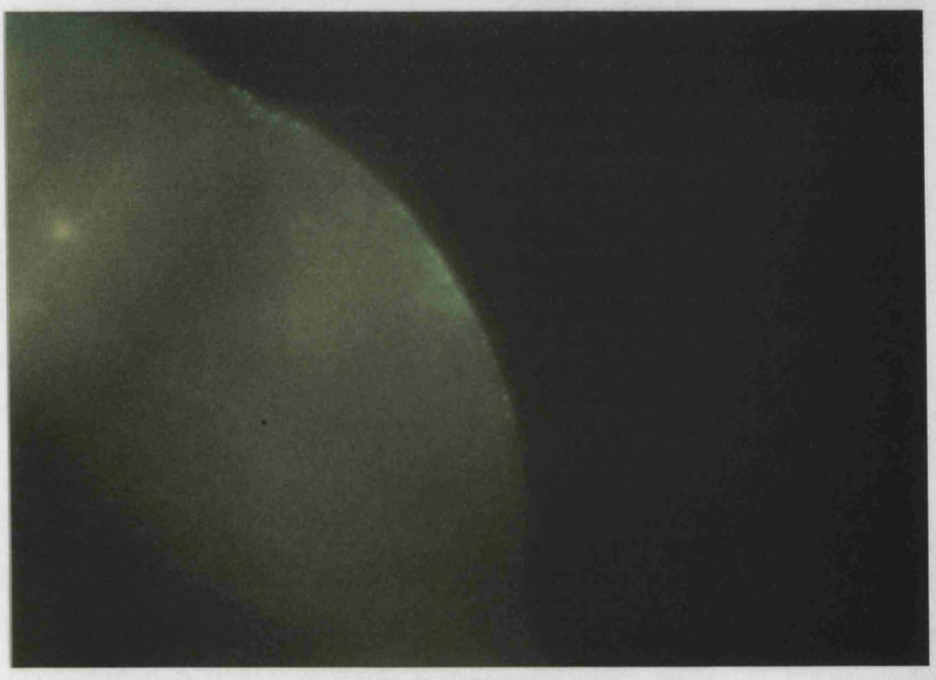

NON — LIGATED
NECK LIGATED + ACETONE
NECK LIGATED + ZR515
0.8
0.4
5
10
15
ABSORBANCE 405nm
FRACTION

Fig 5.9 Staining of PG sections, day W + 2, with G6 MAb and a second Ab-FITC conjugate. (x 700)

Fig 5.10 Staining of intact, unfixed, PG cells (day W + 2) using G6 MAb and a second Ab-FITC conjugate (x 1000).





Chapter VI: Thesis Overview

MAbs were first reported to have been successfully produced in 1975 and since then have become commonplace laboratory reagents. The range of applications for which they have been used as investigative and diagnostic tools is virtually limitless. A large component of the biotechnology industry is now devoted to the production of MAbs chiefly for medically orientated diagnostic kits but also as laboratory tools for affinity purification, immunocytochemistry and immunoassay techniques. There has also been extensive interest in the *in vivo* use of MAb in the therapy of human malignancies, utilising these monospecific Abs to target toxins to the site of tumours and these have been particularly efficacious in the treatment of leukaemias and metastases (eg, Arnon and Sela, 1982). The expansion of this type of application of MAb can be anticipated in the next decade.

Although the generation of MAb is within the scope of many laboratories their application to invertebrate biology has been surprisingly limited and this is probably attributable to economic constraints rather than an indication of the potential these reagents offer to this field of study. However, it is to be hoped that this will change, particularly as invertebrate tissue culture techniques become more widespread as this will provide a basic complement of equipment essential for the generation of hybridomas.

The most critical phase in the production of MAb is the screening procedure which, by definition, will dictate the characteristics of the MAb isolated. This, in conjunction with the type of immunogen used, are the keys to successful MAb generation (see chapter II).

In this thesis two main immunogen preparations were used. First, a pure preparation of a hapten-protein conjugate such as has been used to great effect in the generation of polyclonal antisera. However, the second approach epitomises the strength of MAb in that crude tissue was used as immunogen but, by virtue of several different screening procedures, MAb directed against molecules of potential interest were isolated and used to investigate the biochemical nature and putative functional roles of the original Ags. Without doubt this is the major advantage MAbs offer by comparison with conventional antisera production along with the more practical advantages such as homogeneity of Ab.

First the use of pure immunogens will now be discussed in more detail with reference to the example described in chapter III of this thesis.

Many aspects of polyclonal antisera production, in terms of immunogens, have been directly transferred to hybridoma systems successfully, particularly in the case of low mwt antigens. In these cases the hapten is coupled to a carrier protein to render it antigenic and injected as a pure preparation. This was the strategy employed for MAb production directed against 20-HE with a pure steroid-protein conjugate being prepared to use as the immunogen. It proved to be weakly antigenic, as defined by the number of positive hybridomas generated, and had to be administered in a rigorous immunisation scheme. Consequently it was a costly procedure in terms of the quantity of immunogen which had to be used to generate a limited number of specific MAb secreting cells. Not only were the specific cell lines infrequent, but they were also secreting Ab of low affinity, precluding the use of the MAbs in

practical assay systems. Possible explanations for the low potency of the immunogen include rapid breakdown of the Ag within the animal, its solubility rendering it a poorly recognised Ag or the quality of the protein-steroid conjugate itself in terms of the number of steroid molecules coupled to the protein. The immunisation system was therefore transferred to an *in vitro* culture system in which a suspension of spleen cells was challenged with low doses of the Ag under controlled conditions. This allowed the conjugate to be tested in the absence of many of the variables that are inherent in *in vivo* immunisation such as route of immunisation and rate of breakdown of the immunogen in the bloodstream. This approach proved to be highly successful with many more specific cell lines being isolated even when a fraction of the immunogen dose was used by comparison to the *in vivo* system. Although the MAbs isolated were not of sufficient affinity to be used for assay purposes, this could be achieved if more *in vitro* immunisation were performed. It should also be possible to optimise the culture conditions to allow repeated *in vitro* challenge with immunogen which would generate more IgG Abs by comparison to IgM and could improve the affinity.

The major part of this thesis is devoted to the generation of MAb to a crude tissue preparation and the subsequent partial characterisation of the original Ags using the MAbs as probes. As previously stated, this constitutes a powerful means of isolating hitherto unknown molecules which have the potential to be of structural or functional importance. Chapters IV and V of this thesis describe a library of MAbs generated against the ecdysone secreting PGs. The aim was to screen the fusion wells for specific

MAb directed against cellular components using immunocytochemical approaches and ELISA. The fusion was then re-screened using an ELISA to detect anti-ecdysone MAbs which could be used to detect molecules of putative functional significance. The fusion supernatants were first screened against PG tissue sections to examine the range of staining patterns exhibited by the MAbs. As would be predicted from the injection of intact Pgs into the mouse, the majority of the MAbs were directed against the extracellular connective tissue sheath and the cytoplasm. Anti-nuclear MAbs were relatively infrequent. From the spectrum of MAbs generated there was the potential to characterise molecules within the PG sheath which serves as a barrier between the gland cells and the haemolymph as well as anchoring and supporting the cells within the haemocoel. The MAbs positive in the immunofluorescence screen were re-screened against two types of vertebrate collagen to investigate the possibility that this structural protein could be an important component of the sheath and serve as an indication as to what types of structural proteins are secreted by the PG cells during development. The collagens used in the ELISA screen represented the most common vertebrate fibrillar collagen (type I) and the type IV basement membrane type collagen. This investigation was of significance because the data on insect collagens is sparse and this is the first attempt to use MAbs as probes, with other studies relying on standard chemical characterisation and ultrastructural technique.

The ELISA resulted in the isolation of several MAbs which bound type I collagen with a single MAb binding to both type I and type IV. No MAbs were detected which bound exclusively to type IV collagen. The collagen-binding MAbs were then used to stain collagen

secreting vertebrate cells such as fibroblasts and epithelial cells and extensive intracellular staining was observed by indirect immunofluorescence. The data derived from this study indicates that collagen-like molecules do occur in the PG sheath and that they may share common antigenic sites with collagen and/or pro-collagen molecules, reflecting structural similarity or perhaps homology. The major advantage this type of study has is that these MAbs can now be utilised to isolate the Ag from PG sheath and will be an invaluable tool in the characterisation of these molecules by biochemical techniques. Also they will be useful in the isolation of related molecules from tissues which had previously been considered to contain too little structural protein to be analysed biochemically. Therefore this approach will be useful in studying structural proteins in insects, assessing the extent of homology with vertebrate collagens and gaining insight into the tissue distribution of these molecules, even in tissues which contain trace quantities.

The fifth chapter of this thesis describes the re-screening of the fusion wells generated in response to the PG immunogen and the isolation of a cell line that binds free ecdysone in an ELISA system. This MAb was investigated because, although it was possible that it simply represented a spurious cross reaction by virtue of the original Ag bearing an antigenic determinant(s) which resembles ecdysteroid, it was also conceivable that the Ag within the PG was of functional significance. The MAb was used to probe PG, haemolymph and FB for the presence of the Ag against which the MAb was directed. It proved to occur in all three tissues (but not in ANC) and had a mwt of approximately 200-300 KD. In haemolymph and FB there was also a second immunoreactive molecule which was

detected by the MAb and was of ≤10KD. The 200-300KD molecule appeared to be a protein and was developmentally regulated showing peak levels in PG on day W + 2 (the same stage tissue had been used as immunogen). The low mwt Ag was regulated to a much greater extent and was present at maximal levels on day W + 3 in the FB. The two molecules were investigated under <u>in vitro</u> culture conditions to see if they had any influence on ecdysone secretion rates but in both cases showed no significant effect. This would seem to preclude their involvement in ecdysone secretion and strongly indicates that they do not function as ecdysone precursor binding proteins. Another piece of evidence that suggests that the two mwt Ags are not involved in ecdysone secretion is that neck ligation does not reduce their titers but neck ligation and subsequent ZR515 treatment abolishes the high mwt peak of immunoreactivity and significantly reduces that attributable to the low mwt Ag. It may be hypothesised that JH promotes degradation and/or supresses synthesis of at least the high mwt form and suggests this molecule is of sufficient developmental significance to be hormonally regulated. In conclusion the approach of MAb generation to a crude tissue immunogen has led to the detection of a previously unknown and uncharacterised molecule which seems to be of greater significance than simply a protein which exhibits a common determinant with free ecdysteroid. Not only does the MAb offer a means of fully characterising the original Ag(s) but in general terms this technique should greatly extend our knowledge of the functionally relevant molecules involved in insect development.

It can therefore be seen that hybridoma techniques have a great deal of potential in invertebrate physiology, not only in orthodox immunisation schemes but also in the detection and isolation of

uncharacterised molecules, the functional relevance of which can be probed for using a thoughtful screening assay. Undoubtedly this will provide an invaluable technique in the study of invertebrate systems.

Areas of Future Study

Anti-Ecdysteroid MAbs

The use of *in vitro* immunisation is a promising approach to generating specific, high affinity MAbs by virtue of the numbers of specific hybridomas stimulated using low levels of Ag under standardised conditions. This should provide an invaluable approach to further MAb studies in this area and it is to be hoped that ultimately this will lead to a library of MAbs with defined specificity to be used to study ecdysteroids. Monospecific Abs will probably have most impact on the study of biosynthesis and metabolism which are hampered by low levels of these steroids and the lack of highly specific probes. *In vitro* immunisation could provide an effective means of investigating the possibility of generating specific MAb to molecules differing in only very minor respects from one another and/or which are only weakly antigenic *in vivo*.

Collagen Studies in Insects

From the library of MAbs directed against structural proteins in *Manduca* PG it is now possible to embark on the chemical characterisation of the molecules and to study their homology, if any, to vertebrate collagens and the collagen types identified in

insects to date. The MAb that binds both type I and type IV vertebrate collagen is of potential interest as it suggests that at least two discreet structural proteins are present in the PG sheath. Irrespective of the biochemical nature of these proteins it will be of interest to map their tissue distribution within Manduca. The MAbs will be particularly useful in investigating tissues which contain only low levels of these molecules and would therefore be prohibitively difficult to investigate biochemically.

The G6 Ag(s)

In many ways the study of this protein and a lower mwt cross reactant is just becoming interesting, particularly in the light of the preliminary investigation of neck ligation and JH treatment. The G6 Ag does not appear to be involved in ecdysone synthesis nor is there any strong evidence for it acting as a binding protein. However, it is developmentally regulated, it appears to be depleted in the blood by JH treatment and shows an interesting staining pattern with whole PG, suggesting it to be cytoplasmic and also associated with the gland cell surface. It would be of interest to culture PG in vitro and to determine whether or not the Ag(s) are secreted by the PG under these conditions. Many interesting questions remain to be answered and can be investigated by virtue of the G6 MAb. For instance, what is the distribution of the Ag during early development? The PG of the embryo are relatively large and it would be of interest to know if the G6 Ag(s) is detectable during these early stages. But the main question pertains to the functional nature of these molecules. If G6 is injected into Manduca pupation can be delayed and these investigations could be usefully extended. The in vitro experiments could also be performed

using partially purified, more concentrated Ag(s), perhaps by improving column resolution or by using selective mwt filters. The JH influence on the Ag(s) is really quite fascinating and could be extended to look at the dose response effects of JH treatment on the Ag(s), the influence of JH at different stages of the fifth instar and also the presence of the Ag in the PG and FB throughout the instar.

It seems that the G6 MAb will be an invaluable tool in answering these questions in an attempt to gain insight into the true function of the high mwt protein and the status of the low mwt molecule detected in the blood and FB which is also recognised by the G6 MAb.

Appendix 1

Preparation of Tissue for Electron Microscopy

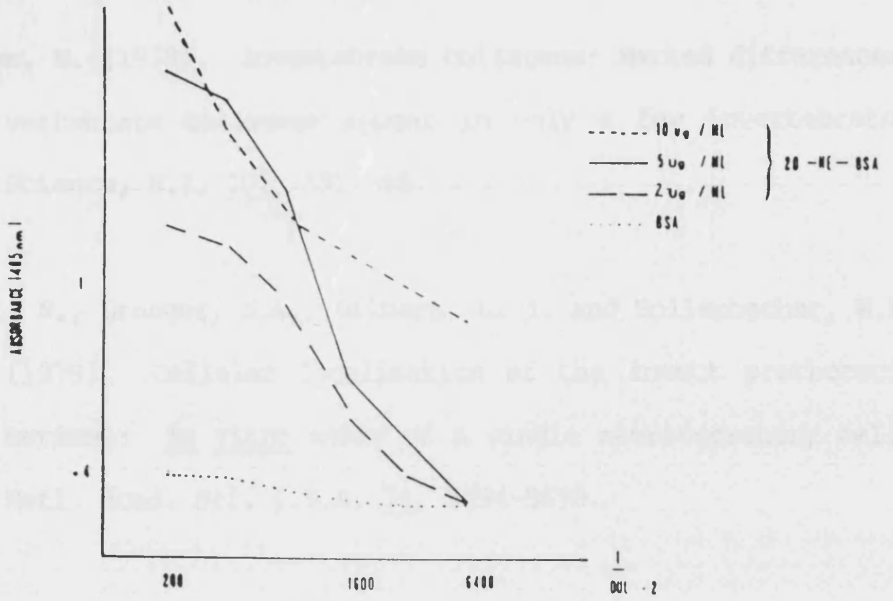
SEM:

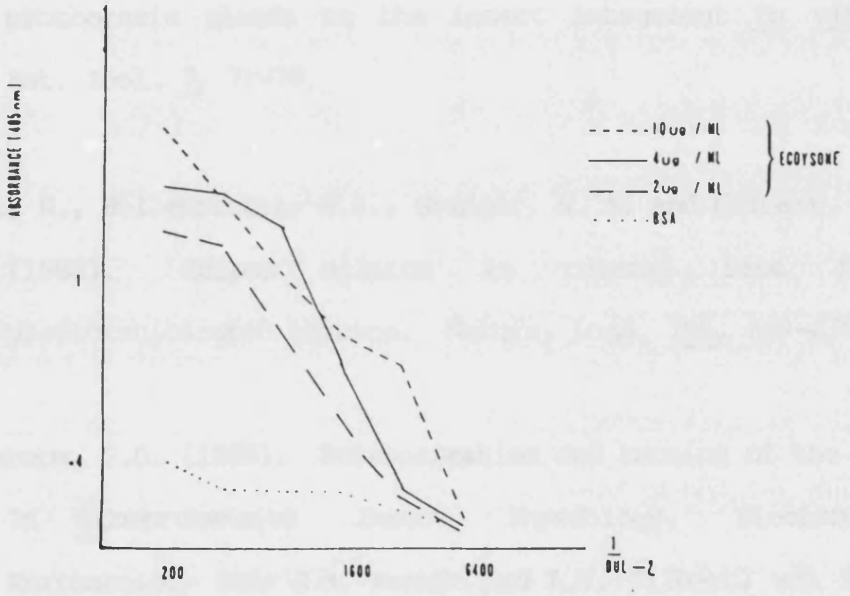
A W + 3 caterpillar was anaesthetised in water for 15 mins and dissected dorsally along the midline and pinned out in a sylguard filled dish (Corning). The preparation was rinsed in Grace's insect culture medium (Flow) and the gut and abdomen removed. After further washing the preparation was immersed in a solution of 1% osmium tetroxide for 1 hour. The fixative was then discarded and the tissue dehydrated through an acetone series and critical point dried. The specimen was sputter coated with gold for 8 mins.

TEM:

PGs were carefully dissected from W + 2 caterpillars and rinsed in Grace's insect culture medium (Flow) and fixed overnight in 4% glutaraldehyde in 0.1 m cacodylate buffer. The tissue was washed in three changes of distilled water and transferred to a 1% solution of osmium tetroxide for 1 hour. The washing step was repeated. The PGs were placed in a 1% aqueous solution of uranyl acetate for 30 mins, washed twice in distilled water and dehydrated through an acetone series. The tissue was placed in EM blocks containing resin/acetone (1:1, v/v) and left overnight at room temperature. The resin/acetone mixture was replaced with 100% resin and left overnight. The resin was changed and left overnight. The blocks were then hardened and sectioned in a standard manner. Sections on grids were stained for 3 mins in a 1% solution of aqueous uranyl acetate, washed in three changes of distilled water and air dried.

APPENDIX 2





APPENDIX 3

There also remains the possibility that rather than the coupling ratio being over estimated that the quality of the oxime produced had not been adequately assesed. Perhaps nuclear mass resonance analysis would have given a more definitive answer to whether the oxime had been successfully synthesised. In future conjugate synthesis this would be a prepherable technique to the U.V analysis used in this study.

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